The role of cytosolic calcium signaling in beneficial and pathogenic interactions in *Arabidopsis thaliana*

seit 1558

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

Vorgelegt dem

Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller-Universität Jena

von Master of Science in Agriculture (Plant Pathology)

Joy Michal Johnson

geboren am 25.05.1971 in Thiruvananthapuram, Kerala, India

Gutachter

1. **Prof. Dr. Ralf Oelmüller**, Department of Plant Physiology, Institute of General Botany and Plant Physiology, Friedrich-Schiller University Jena, Germany.
2. **Prof. Dr. Philipp Franken**, Department of Molecular Phytopathology, Institute of Biology, Humboldt University Berlin, Germany.
3. **Prof. Dr. Natalia Requena**, Department of Molecular Phytopathology, Botanical Institute, Karlsruhe Institute of Technology Karlsruhe, Germany.

Datum der Verteidigung : 28-01-2014

Table of contents

Abbreviations

Ab	*Alternaria brassicae*
ABA	abscisic acid
ab-cycam	cytosolic calcium mutant to Ab-CWE
aGLS	aliphatic glucosinolate
3-AT	3-aminotriazole
bMAMPs	beneficial microbe-associated molecular patterns
$[Ca^{2+}]_{cyt}$	cytosolic calcium concentration
cis-OPDA	cis-12-oxo-phytodienoic acid
Col-0	Arabidopsis ecotype Columbia
CWE	cell wall extract
dai	days after inoculation
EMS	ethane methyl sulfonate
EPM	exudates preparation from mycelium
EPS	exudates preparation from germinating spores
FW	fresh weight
HL medium	Hoagland medium
iGLS	indolic glucosinolate
(+)-7-*iso*-JA-Ile	(+)-7-*iso*-jasmonoyl-l-isoleucine
JA	jasmonic acid
JA-Ile	jasmonic acid-isoleucine conjugate
kD	kilo dalton
KM	Kaefer Medium
La-*er* (L*er*-0)	Arabidopsis ecotype Landsberg erecta
MAMPs	microbe-associated molecular patterns
Mb	millions of base pairs
mg	milli gram
min	minute
MS medium	Murashige and Skoog medium
m/z	mass by charge ratio
PAMPs	pathogen-associated molecular patterns
PDA	potato dextrose agar
PDI	percentage disease index
Pi	*Piriformospora indica*
pi-cycam	cytosolic calcium mutant to Pi-504
ROS	reactive oxygen species
RRTF1	redox responsive transcription factor1
SA	salicylic acid
WDE	water diffusible extract
WT	wild-type

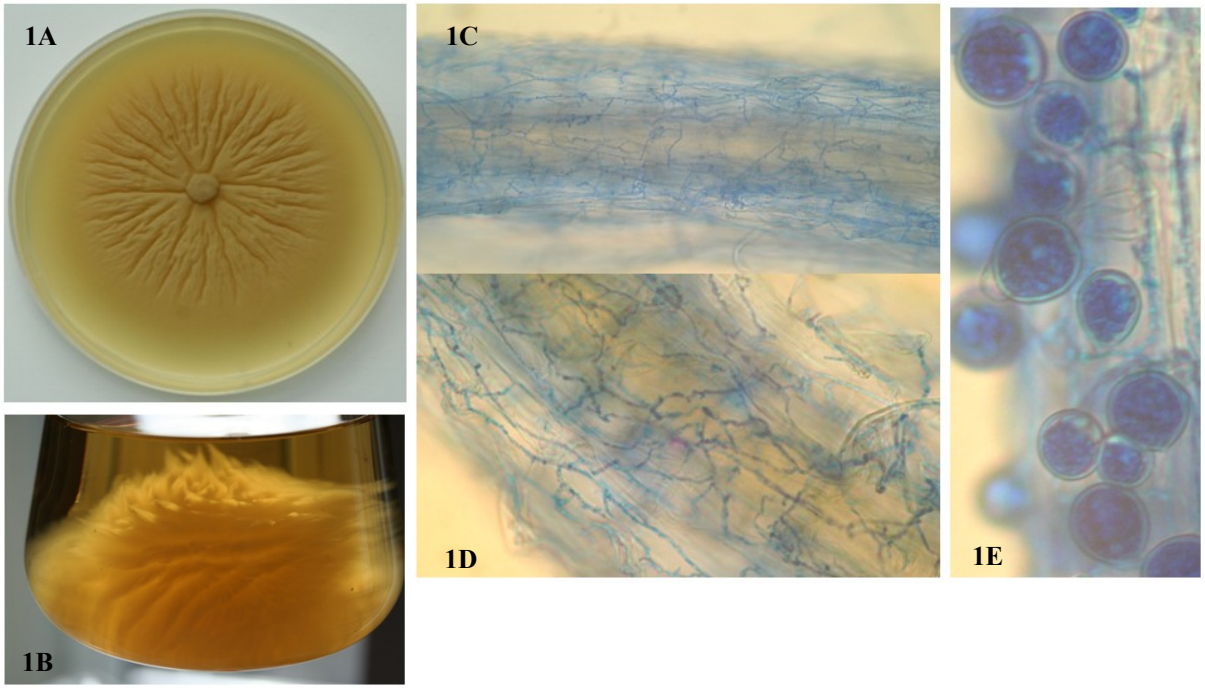
1. Introduction

Plants continuously interact with a wide variety of microorganisms in their natural environment with interactions being non-host, beneficial or pathogenic to the plants. Symbiosis refers to organisms living together, whether the interaction is mutualistic (beneficial for both partners), commensalic (beneficial for the microbe, while the host species is neither positively nor negatively affected) or parasitic (the host is parasitised or deprived at the expense of the fungus) (Kogel *et al*. 2006; Johnson and Oelmüller 2009). The well studied endosymbiotic mutualistic interactions are Rhizobium bacteria in legumes and arbuscular mycorrhizal fungi (AMF) in vast majority of terrestrial plants (Parniske 2008; Oldroyd *et al*. 2011; Oldroyd 2013). Both endosymbioses result in an improved uptake and assimilation of nutrients (e.g., nitrogen, phosphorus, sulphur and iron.) from the soil by the plant partner and in exchange, allow the endosymbiont to obtain the photosynthetically fixed carbon sources (e.g. carbohydrate) necessary for their survival and propagation. Plant endosymbioses are characterized by the penetration of living plant cells by a microbial symbiont, followed by a period during which the symbiont lives partially or entirely within plant cells by benefiting each other (Parniske 2008). Mutualistic interactions between endosymbionts and plant roots have been a fundamental pre-requisite for evolution and biodiversity of land plants (Weiß *et al*. 2011). Most of the terrestrial plants (> 80 %) have established a symbiotic association with AMF, which is an intricate association of plant roots with fungi belonging to the order Glomales of the Glomeromycota. The main obstacles in the molecular analysis of beneficial plant/microbe interactions are the lack of genomic information for most plant species that form either bacterial or fungal symbiosis, obligate nature of AMF and the bacterial nodulation is restricted mostly to legumes. Moreover, both rhizobial and mycorrhizal colonization are absent in the genetically well characterized *Arabidopsis thaliana*. But the discovery of axenically cultivable plant growth promoting root endophyte, *Piriformospora indica* (Verma *et al*. 1998; Varma *et al*. 1999), and its ability to form endosymbiosis with many host plants including *A. thaliana* have opened a new vista to study the early and late molecular and physiological events associated with the beneficial plant-fungus interaction.

1.1 *Piriformospora indica* - morphology, taxonomy and root colonization pattern

P. indica (Verma, Varma, Kost, Rexer & Franken) grows on a wide range of synthetic and complex media including Kaefer medium (KM) (Figures 1A-B) which is rich in sugar and

protein. Young hyphae and mycelia are hyaline, branched, mostly submerged into the substratum, thin walled, often intertwined and show a nodose to coralloid shape with dense bodies when it colonizes on the root (Figures 1C-D). Ultrastructure examination of the septal pore reveals that it has dolipore septation which is a unique feature of basidiomycete fungi (Varma *et al*. 2012). The anamorph (asexual) stage is characterized by the formation of chlamydospores at the tip of the hyphae singly or in clusters. Chlamydospores are distinctive due to their pear-shape (Figure 1E) and it contains 8 to 25 nuclei. Neither clamp connections nor sexual structures are observed in *P. indica* unlike other basidiomycetes; therefore the teleomorph (sexual) stage of the fungus is unknown. Molecular phylogenetic analyses reveal that *P. indica* is a member of basidiomycetous order Sebacinales (Agaricomycetes, Basidiomycota) (Weiß *et al*. 2011). The molecular phylogeny also shows that *P. indica* is closely related to *Rhizoctonia solani* Kuhn and *Thanatephorus praticola* (Kotila) Talbot (Weiß *et al*. 2011). Cell biology studies in *Arabidopsis* show that the fungus colonizes every parts of the root, e.g. root tip and meristematic, elongation and maturation zones, and its colonization is restricted to epidermis and cortex (Figures 1F-J). The fungus enters living root cells by direct penetration, colonizes in the root epidermal and cortical tissues, and grows inter- and intracellularly forming pear- shaped chlamydospores after 14 days of colonization (Figures 1C-E). The fungus has early biotrophic interaction and is followed by a cell death-dependent colonization phase in the host cells (Deshmukh *et al*. 2006; Zuccaro *et al*. 2011; Qiang *et al*. 2012b).



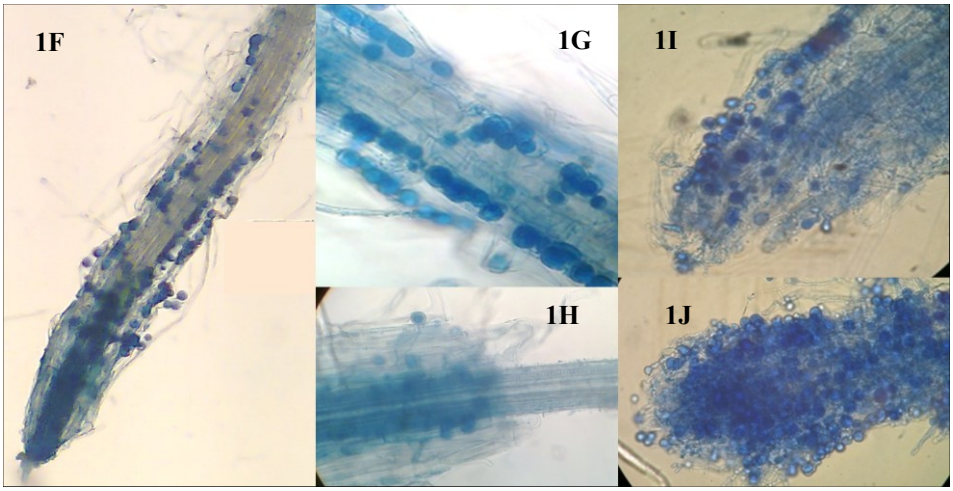


Figure 1. *P. indica* growth and its colonization in *A. thaliana* root. Four-week old *P. indica* on KM plate (A); 16-18-day old *P. indica* growth in KM broth (B); thin walled hyphae on colonized root (C); nodose to coralloid shaped hyphae with granulated dense bodies before sporulation (D); pear-shaped chlamydospores after 18 days of colonization with the root (E); external and internal colonization of the fungus on the root tip, and meristematic, elongation and maturation zones of the root (F); inter- and intra-cellular fungal colonization with chlamydospores inside the root cells (G); restriction of the fungal colonization to epidermis and cortex of the root and the stelar region is free of the fungal colonization (H); the fungal colonization on the root tip (I) and the heavy sporulation of the fungus on the root tip (J).

1.2 *P. indica* - a plant growth promoting root endophytic fungus

P. indica, the novel endophytic root-colonizing fungus, was isolated from the rhizosphere of xerophytic woody shrubs e.g. *Prosopis juliflora* and *Zizyphus nummularia* in the Thar desert of Noth-Western India (Verma *et al*. 1998). The fungus lacks host specificity and possesses a broad host spectrum including bryophytes (*Aneura pinguis*), pteridophytes (*Pteris ensiformis*), gymnosperms (*Pinus halepensis*) and a large number of angiosperms, and positively affects different aspects of plant performance (c.f. Oelmüller *et al*. 2009; Franken 2012; Qiang *et al*. 2012a; Varma *et al*. 2012). Similar to AMF, the beneficial interaction of *P. indica* with a large number of horticulturally and agriculturally important crops ultimately leads to growth promotion (Figures 2A-D) and enhanced resistance/tolerance against biotic and abiotic stress (Varma *et al*. 1999, 2012; Sherameti *et al*. 2005; Waller *et al*. 2005; Serfling *et al*. 2007; Shahollari *et al*. 2007; Oelmüller *et al*. 2009; Sun *et al*. 2010; Johnson *et al*. 2011b; Molitor *et al*. 2011; Franken 2012; Kumar *et al*. 2012). Once inside the roots, the fungus gets access to photoassimilates and other plant nutrients, which further promotes colonization and proliferation, and significantly enhances plant growth (Oelmüller *et al*. 2009). Plants benefit from this relationship by reprogramming plant transcriptomes, proteomes and metabolomes that are directly or indirectly involved in phytohormone

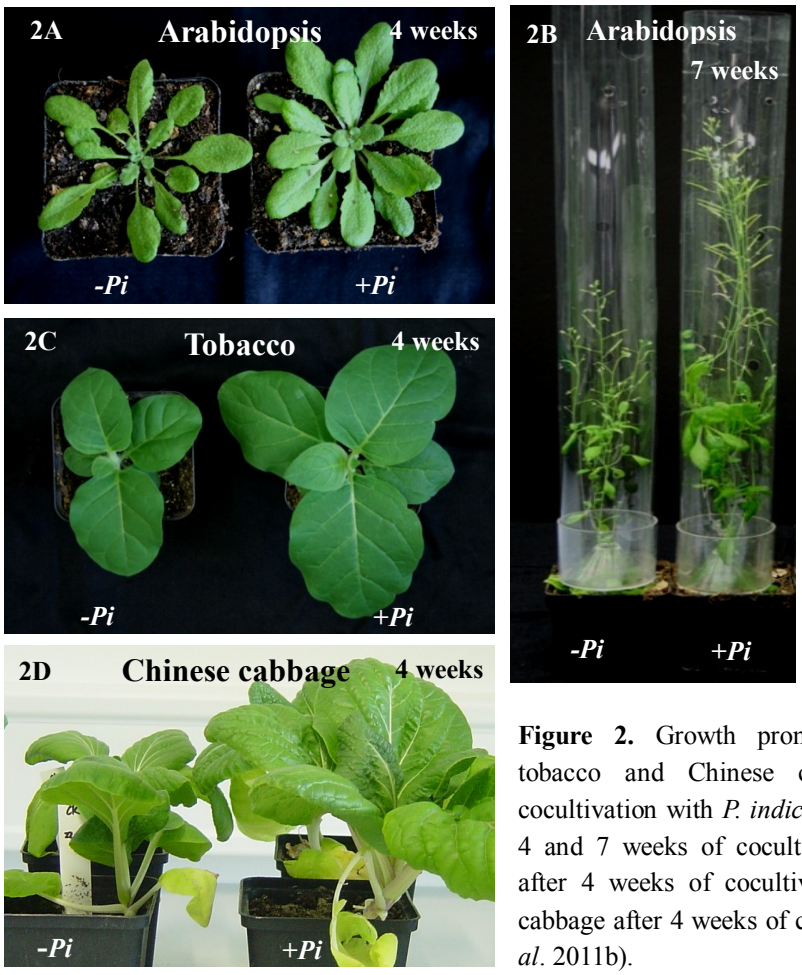


Figure 2. Growth promotion of Arabidopsis, tobacco and Chinese cabbage in soil after cocultivation with *P. indica (Pi)*. Arabidopsis after 4 and 7 weeks of cocultivation (A, B); tobacco after 4 weeks of cocultivation (C) and Chinese cabbage after 4 weeks of cocultivation (Johnson *et al*. 2011b).

synthesis and signaling, increased nutrient uptake and growth, early flowering, enhanced seed production, and protection against drought, salinity and phytopathogens (cf. Oelmüller *et al*. 2009; Franken 2012). The modulation of gene expressions, proteins and metabolites helps both partners to keep the interaction mutually beneficial. Recent establishment of a transformation system and the full genome sequence of the fungus will likely stimulate great progress towards further functional analysis of the endosymbiont. The fungus has a 25-Mb genome with 11,768 putative protein encoding genes which also include the genes responsible for mutualistic interaction, and the early biotrophic and late necrotrophic phases of the fungus (c.f. Zuccaro *et al*. 2011).

1.2.1 Nutrient uptake in *P. indica*-colonized plants

One common feature of symbiotic associations is the ability of the microbial endosymbionts to assimilate nitrogen and phosphorus that limit plant growth and to trade these nutrients with the plant in exchange for a carbon source derived from photosynthesis (Parniske 2008; Oldroyd 2013). It is reported that *P. indica* helps to transfer nitrogen and phosphorus from the

environment to the host plants (Sherameti *et al*. 2005; Yadav *et al*. 2010). The growth promotion in *P. indica*-colonized *Arabidopsis* and tobacco seedlings is attributed to the upregulation of *nitrate reductase* (*Nia2*), a gene involved in nitrate uptake, and the starch-degrading enzyme, glucan-water dikinase (*SEX1*), involved in carbohydrate metabolism in roots and shoots (Sherameti *et al*. 2005). Shahollari *et al*. (2007) could link growth promotion of Arabidopsis seedlings with a massive uptake of radiolabeled phosphorus from the growth medium. Uptake and transport of phosphorus is also stimulated by the fungus in the colonized roots of maize (Yadav *et al*. 2010). Recently *P. indica* phosphate transporter (PiPT) protein is purified, crystalized and characterized (Pedersen *et al*. 2013).

1.2.2 Role of phytohormones in mutualistic interaction of *P. indica* with plants

Auxin, cytokinin, ethylene, abscisic acid and gibberellins are the main phytohormones involved in the development of new organs or promotion of growth in plants. Many beneficial interactions interfere with auxin metabolism and its functions in the plants which is well established in root nodule forming bacteria (Perrine-Walker *et al*. 2010), mycorrhizal fungi (Ludwig-Müller and Güther 2007) and plant-growth promoting rhizobacteria (Contesto *et al*. 2010). It is reported that auxin, cytokinins and gibberellins plays a crucial role in *P. indica*-induced growth promotion in Arabidopsis, barley and Chinese cabbage (Sirrenberg *et al*. 2007; Vadassery *et al*. 2008; Schäfer *et al*. 2009a, b; Lee *et al*. 2011). Growth promotion of Chinese cabbage and barley seedlings is attributed to the increased auxin level in the colonized roots, even though the auxin level in the leaves is not affected by the fungus (Schäfer *et al*. 2009b; Sun *et al*. 2010; Lee *et al*. 2011). Large scale microarray analysis of *P. indica*-colonized Arabidopsis roots did not reveal many auxin-related genes as a target of the fungus. Mutants with impaired auxin levels (*ilr1-1*, *nit1-3*, *tfl2*, *cyp79 b2b3*) responded to *P. indica*, revealing that severe alterations in auxin homeostasis in Arabidopsis do not prevent the growth response to *P. indica* (Vadassery *et al*. 2008). However, cytokinin, another phytohormone involved in cell division, vascular development, sink/source relationships, apical dominance, stress tolerance and leaf senescence is significantly increased in *P. indica*-colonized Arabidopsis roots (Vadassery *et al*. 2008). This is evident from the fact that different cytokinin receptor genes viz. *CRE1, AHK2, and AHK3* and *cytokinin-responsive gene ARR5*, and *trans*-zeatin cytokinins biosynthesis genes are significantly upregulated in the colonized roots. Mutant studies where *trans*-zeatin biosynthesis is impaired, showed no growth promotion to the fungus indicating that the biosynthesis of *trans* zeatin- but not *cis*

zeatin- type cytokinins are required for *P. indica*-induced growth promotion in Arabidopsis (Vadassery *et al*. 2008). Another phytohormone, ethylene, typically inhibits plant growth, but *P. indica* inhibits ethylene biosynthesis and its signaling, which could contribute to plant growth promotion in tobacco and barley (Barazani *et al*. 2007; Schäfer *et al*. 2009a, b). Ethylene also has a key role in the beneficial interaction between *P. indica* and Arabidopsis. The growth of *etr1*, *ein2* and *ein3/eil1* impaired in ethylene biosynthesis and activation of ethylene responsive genes, for example, *ETR1, EIN2 and EIN3 / EIL1* are not promoted or even inhibited by the fungus (Camehl *et al*. 2010). Interestingly, Schäfer *et al*. (2009a) reported the positive role of gibberellins in the mutualistic association of *P. indica* with barley. Other phytohormones synthesized or manipulated by the root endophyte include abscisic acid (ABA) and brassinosteroids (Schäfer *et al*. 2009a; Vadassery *et al*. 2008; Camehl *et al*. 2011). It seems that the whole orchestra of phytohormones and its signaling networks are involved in generating compatible endosymbiosis between the fungus and plants (Franken 2012).

1.2.3 Role of *P. indica* in tolerance to abiotic stress

Plants are exposed to a wide variety of dynamic environments including biotic and abiotic stress in their natural growth habitat. The drought tolerance in the *P. indica*-colonized plants is associated with the activation of antioxidant enzymes, the upregulation of drought-related genes, e.g. *DREB2A*, *CBL1*, *ANAC072* and *RD29A*, and rapid accumulation of the plastid-localized Ca^{2+}-sensing (CAS) protein (Sherameti *et al*. 2008a; Vadassery *et al*. 2009b; Sun *et al*. 2010). The expressions of *MDAR2* and *DHAR5*, two ascorbate reductase genes having antioxidant and reactive oxygen species (ROS) scavenging functions, are upregulated in the roots and shoots of colonized Arabidopsis seedlings exposed to drought. Moreover, the interaction studies with *mdar2* and *dhar5* show that MDAR2 and DHAR5 are crucial to maintain the interaction in a mutualistic state (Vadassery *et al*. 2009b). The activities of antioxidant enzymes, e.g. peroxidases, catalases and superoxide dismutases, are significantly high in *P. indica*-colonized Chinese cabbage plants which are exposed to drought stress (Sun *et al*. 2010). The fungus also reduced the accumulation of stress molecule malondialdehyde, the drought-induced decline in the photosynthetic efficiency, and the degradation of chlorophylls and thylakoid proteins in the colonized plants (Sun *et al*. 2010). The fungus also protected the plants from the moderate levels of NaCl stress (100 mM) in barley (Waller *et al*. 2005; Baltruschat *et al*. 2008) and wheat (Zarea *et al*. 2012).

1.2.4 Activation of defense responses by *P. indica*

Endosymbiosis between the interacting partners requires a sophisticated balance between defense and growth responses in the host plant, and the nutrient demand of the fungus (Johnson and Oelmüller 2009). *P. indica*-colonized plants acquire resistance against foliar pathogens e.g. powdery mildew infection in barley, wheat and Arabidopsis (Serfling *et al*. 2007; Stein *et al*. 2008; Molitor *et al*. 2011), and a variety of root diseases in wheat, barley, maize and tomato (Serfling *et al*. 2007; Stein *et al*. 2008; Kumar *et al*. 2009). It is reported that *P. indica* moderately upregulates the expression of various defense genes during the early stages of colonization, later they are downregulated as the interaction progresses (Camehl *et al*. 2010). A number of mutants have been isolated, where the interaction is shifted from mutualism to parasitism (cf. Johnson and Oelmüller 2009). *PYK10*, a defense gene involved in the synthesis of a root- and hypocotyl-specific beta-glucosidase/myrosinase located in the endoplasmic reticulum, is induced significantly in the colonized roots of Arabidopsis whereas, *pyk10* and *nai1* or *pii-4* with reduced or no beta-glucosidase/myrosinase do not show growth promotion after colonization with the fungus (Sherameti *et al*. 2008b). The above mutants show upregulation of the plant defensin 1.2 (*PDF1.2*) involved in plant defense, which further restrict the colonization of *P. indica* in the root. PYK10 exhibits striking sequence similarities to PEN2, a glycosyl hydrolase, which restricts the entry of powdery mildew fungi into Arabidopsis leaf (Lipka *et al*. 2005). The fungal colonization systemically induces resistance (induced systemic resistance - ISR) against foliar pathogens indicating that there must be an efficient information flow from the roots to the shoots (Oelmüller *et al*. 2009; Molitor *et al*. 2011). *P. indica*-/primed plants help the shoots become preconditioned to develop an enhanced capacity for activating defense responses prior to infections by pathogens via. jasmonic acid (JA) and ethylene signals from the roots and thereby resisting different foliar pathogens (Stein *et al*. 2008; Molitor *et al*. 2011). Different JA (*VSP*, *PDF1.2* and *LOX2*) and ethylene (*ERF1*) signaling but not salicylic acid (SA) signaling (*PR1* and *PR5*) genes are upregulated in the *P. indica*-primed plants challenged with the powdery mildew fungus (Stein *et al*. 2008). The mechanisms of *P. indica*-induced resistance is similar to the well characterized ISR described for plant growth promoting rhizobacteria-colonized plants (van Wees *et al*. 2008; Jacobs *et al*. 2011).

1.2.5 The role of secondary metabolites in *A. thaliana-P. indica* interaction

The most important secondary metabolites synthesised by the members of brassicales are

aliphatic glucosinolate (aGLS) from methionine, and indolic glucosinolates (iGLS) and camalexin from tryptophan (Sonderby *et al*. 2010). CYP79B2 and CYP79B3 are two functionally redundant cytochrome P450 enzymes which convert tryptophan into indole-3-acetaldoxime (IAOx), an intermediate for the biosynthesis of iGLS and camalexin, from indole-3-acetic acid (IAA). The R2R3 MYB transcription factors MYB28, MYB29 and MYB76 are the positive regulators of the aGLS biosynthesis (Sonderby *et al*. 2010). Constitutive production of defense compounds and its induction during pathogenesis are important for plant defense (Halkier and Gershenzon 2006). Necrotrophic pathogens induce the synthesis of the aliphatic and indolic glucosinolates, and camalexin in Arabidopsis (Su'udi *et al*. 2011; Foley *et al*. 2013; Wang *et al*. 2013). Different PEN mutants accumulated more iGLS and camalexin in response to powdery mildew fungus *Blumeria graminis hordei* and the necrotrophic fungus *Plectosphaerella cucumerina* infections (Bednarek *et al*. 2009). The protective role of aGLS is shown against *Sclerotinia sclerotiorum* using *myb28* (Stotz *et al*. 2011). Nongbri *et al*. (2012) reported that iGLS restricts the colonization of *P. indica* in Arabidopsis.

1.3 Early signal transductions in *A. thaliana* and *P. indica* interaction

A. thaliana-P. indica is an excellent model system for the elucidation of the early signaling events and molecular mechanisms involved in the host recognition and subsequent beneficial effects. The complex beneficial interaction between root and fungus necessitates continuous recognition and signal exchange between both partners (Oelmüller *et al*. 2009). Soluble compound(s) released/exudated from the fungus, extracellular proteins from the plant, receptor-like kinases, phospholipids, phosphorylation cascades, Ca^{2+} signaling in both cytoplasm and nucleus, mitogen activated protein kinase (MAPK) activation as well as the stimulation of specific transcription factors are involved in this beneficial interactions (Oelmüller *et al*. 2009). The proteomic approaches combined with ethane methyl sulfonate (EMS) mutagenesis has led to the identification of several *P. indica* responsive Arabidopsis proteins like a MATH (meprin and tumor necrosis factor receptor-associated factor (TRAF) homology) domain containing protein (Oelmüller *et al*. 2005), a leucine rich repeat protein 2 LRR2 (Shahollari *et al*. 2007), PYK10, a β-glucosidase located in the endoplasmic reticulum (Sherameti *et al*. 2008b), ROP, a RHO-related GTPase (Venus and Oelmüller 2012), and signaling cascades involving cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), phospholipids, ROS and MAPK (Vadassery *et al*. 2009a). Two leucine rich repeat proteins LRR1 and LRR2, located in lipid

rafts/plasmamembrane microdomains of the roots, are required for the mutualistic interaction as their inactivation completely prevents the beneficial interaction (Shahollari *et al*. 2007). Partial inactivation of a sphingosine kinase, which is required for the biosynthesis of sphingolipids, also affects the *A. thaliana-P. indica* interaction (Shahollari *et al*. 2007). Downstream signaling requires phospholipids, and activates a kinase cascades which includes 3-phosphoinositide-dependent protein kinase1 (PDK1), oxidative signal-inducible1 (OXI1), and MAPKs (Rentel *et al*. 2004; Vadassery *et al*. 2009a; Camehl *et al*. 2011). These proteins and signaling molecules are expressed and induced during early interaction stages, and are crucial for the growth promotion by the fungus.

1.4 Plant recognition of *P. indica* and other microbial factors

Endosymbionts release various factors necessary for its recognition by plant cells to initiate mutualistic interaction. For successful endosymbiosis, a molecular dialogue is essential which is a two way process involving the host plant and the microbial symbionts. In rhizobial symbiosis, roots release flavonoids while the bacteria release nodulation (Nod) factors (lipochitooligosaccharide) (Ehrhardt *et al*. 1996) to initiate signaling cascades needed for nodulation process. In AM symbiosis, plants release strigolactones, which acts as branching factor for fungal hyphae (Akiyama *et al*. 2005), while the fungus releases the Myc factor (lipochitooligosaccharide) (Kosuta *et al*. 2003; Maillet *et al*. 2011). In pathogenic fungi these signaling molecules are chitin, glucan or protein by nature, which activates defence genes on recognition by plant cells (cf. Lecourieux *et al*. 2006). The receptor-mediated recognition of PAMPs/MAMPs (pathogen-/microbe-associated molecular patterns) activates the basal defense of plants against invading pathogens or microbes. Activation of innate immune response or basal defence does not prohibit colonization of microbes but only controls its spread so that the interacting microbe will be recognised either as pathogen or as symbiont by the host cell (Johnson and Oelmüller 2009). Downstream of the receptor perception is the chain of signaling events leading to defense-related genes activation including an oxidative burst and MAPK activation (Blume *et al*. 2000). During compatible interactions, pathogen-derived effector/virulence molecules suppress PAMP-induced defense responses, and enable the pathogen to overcome basal resistance and to successfully infect the plant (Jones and Dangl 2006). Component(s) from the cell wall extract of *P. indica* (Pi-CWE) also promotes the plant growth as the fungus does (Vadassery *et al*. 2009a) (Figure 3). Pi-CWE and the fungus also modulate a similar set of genes in *Arabidopsis* roots which also include genes

with Ca^{2+} signaling-related functions. Therefore, Ca^{2+} is likely to be an early signaling component in the mutualistic interaction between *P. indica* and Arabidopsis (Vadassery *et al*. 2009a).

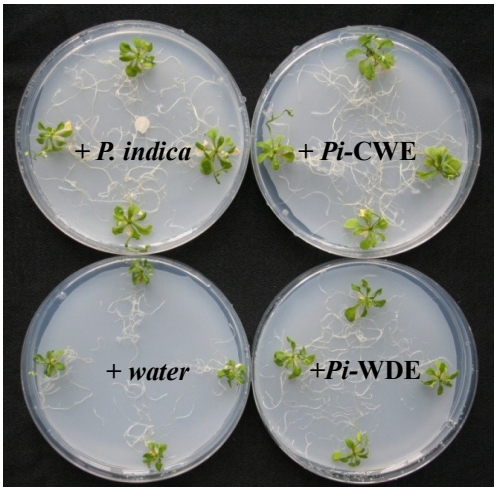


Figure 3. Growth promotion of *A. thaliana* seedlings after 14 days of cocultivation on PNM medium with the *P. indica* plug and treated with its cell wall extract (Pi-CWE) and water diffusible extract (Pi-WDE). Water is used as control (Johnson *et al*. 2013b).

1.5 Calcium signaling - a ubiquitous cellular second messenger

Plants have evolved very effective mechanisms to perceive, transduce and respond to a wide variety of biotic and abiotic signals by modulating intracellular or cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) (c.f. Sanders *et al*. 2002; Lecourieux *et al*. 2006; Dodd *et al*. 2010; Kudla *et al*. 2010; Reddy *et al*. 2011). Perception of stimuli through membrane bound receptors results in generation of Ca^{2+}, a unique second messenger of signal transduction in cells, which convey signals to the cellular machinery to initiate specific signaling events leading to a specific biological response (Reddy and Reddy 2004; Reddy *et al*. 2011). The cellular Ca^{2+} levels are tightly regulated and even a small change in the cytosolic concentration provides information for protein activation and signaling (Sanders *et al*. 2002). Ca^{2+} signaling is composed of a receptor, a system for generating the transient or slow increase in $[Ca^{2+}]_{cyt}$ in response to a stimulus, recognition of the Ca^{2+}-signature by sensors, transduction of the signature message to targets and other cellular systems responsible for returning $[Ca^{2+}]_{cyt}$ to its prestimulus level (Hetherington and Brownlee 2004, Reddy and Reddy 2004). Changes in $[Ca^{2+}]_{cyt}$ occur in response to many abiotic signals such as light, low and high temperature, touch, drought, oxidative stress and phytohormones, and the biotic signals including bacterial/fungal/-oomycete PAMPs, Nod factor and Myc factor (c.f. Sanders *et al*. 2002; Lecourieux *et al*. 2006; Dodd *et al*. 2010; Reddy *et al*. 2011). $[Ca^{2+}]_{cyt}$ elevation is due to an uptake of Ca^{2+} from the extracellular medium, or by Ca^{2+} mobilization from organelles, and/or by both (Lecourieux *et al*. 2006). Recombinant aequorin technology based on bioluminescence is used

to measure $[Ca^{2+}]_{cyt}$ in plant cells (Knight *et al*. 1991, 1997) (Figure 4).

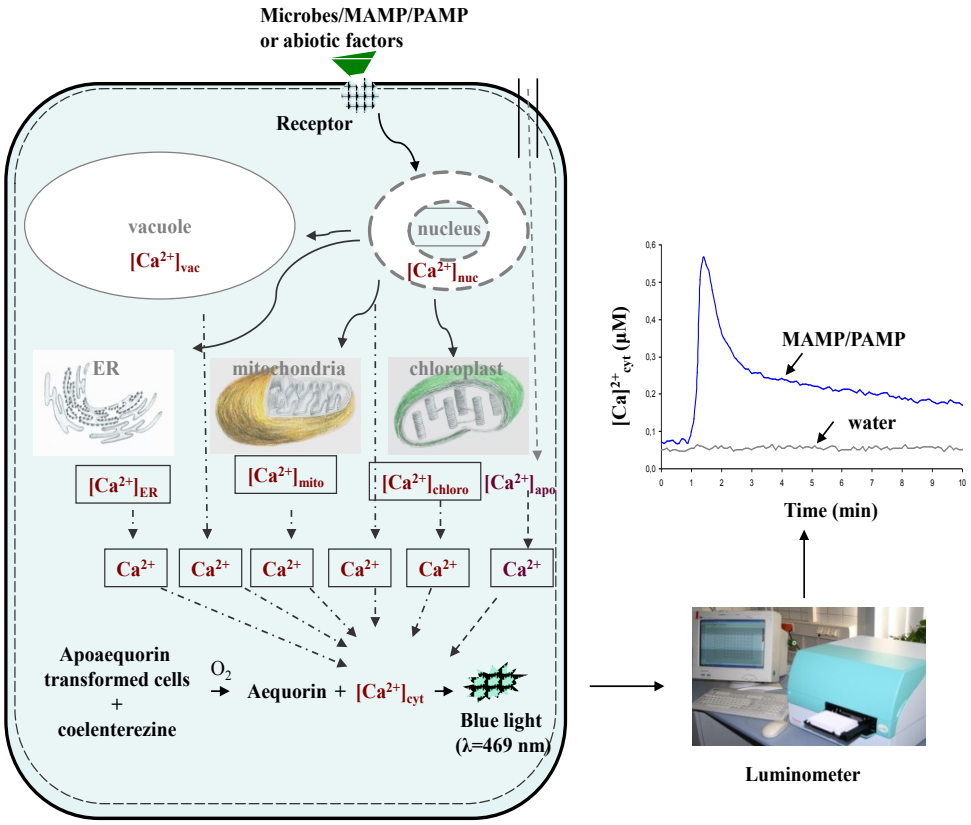


Figure 4. Recombinant aequorin technology to measure $[Ca^{2+}]_{cyt}$ elevation in plant cells. Apoaequorin is expressed in all cells of Arabidopsis plant by stable transformation techniques (pMAQ2). By incubating the cells or tissues with 5-10µM coelenterazine, the functional aequorin is reconstituted in all cells in the presence of oxygen. When biotic agents and/or its molecules or abiotic factors are perceived, the $[Ca^{2+}]_{cyt}$ is released from the apoplast (apo) and different internal Ca^{2+} stores, for example, nucleus (nuc), vacuoles (vac), endoplasmic reticulum (ER), chloroplasts (chloro) and mitochondria (mito) into the cytoplasm through different calcium permeable channels, pumps, transporters and exchangers. This free Ca^{2+} binds to the functional aequorin and emits blue light which is captured by a high throughput luminometer as relative light units (RLUs). RLUs are converted into cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) using the formula (Johnson *et al*. 2011a).

1.5.1 Role of Ca^{2+} signaling in endosymbiotic plant-fungus interactions

As both AM fungi and *P. indica* form mutually beneficial symbiotic association with many plants, the interaction should be based on general recognition and signaling processes. (cf. Oelmüller *et al*. 2009; Smith and Smith 2011). The complex cellular interaction between root and fungi necessitates continuous recognition and signal exchange between both partners (Bonfante and Requena 2011). Similar to the rhizobial nodulation in legumes, the interaction of AMF and *P. indica* with roots results in better plant performance through sequential cytoplasmic and nuclear Ca^{2+} elevations (Navazio *et al*. 2007; Kosuta *et al*. 2008; Vadassery

et al. 2009a; Oldroyd *et al*. 2011). Large scale mutant screenings revealed that the *Does Not make Infection* (*DMI*) genes are the most important prerequisite genes for successful rhizobial and AM symbioses (Parniske 2008; Oldroyd *et al*. 2011). After the perception of Nod and Myc factors by their respective receptors, three DMI genes e.g. *DMI1*, a putative cation channel, *DMI2/NORK/SYMRK* a receptor-like kinase with LRR domains and *DMI3* a chimeric Ca^{2+}/calmodulin-dependent protein kinase (CaMK), with Ca^{2+} signaling functions play a significant role in these symbiotic interactions (c.f. Singh and Parniske 2012; Oldroyd 2013). The *DMI* genes that are essential for rhizobial Nod factor signal transduction are also required for the symbiosis with AM fungi and are referred as *SYM* genes, and mutations in all these genes fail to allow entry of the fungus into the cortex (Catoira *et al*. 2000). Diffusible myc factor is involved in a variety of host responses including the activation of *SYMBIOSIS* (*SYM*) genes (Kosuta *et al.* 2003; Singh and Parniske 2012), the stimulation of lateral root branching (Akiyama *et al*. 2005) and the activation of starch-related metabolic pathways (Gutjahr *et al.* 2009) in *M. trunculata*. Myc factor does not induce any defense responses like ROS accumulation and cell death in host cells (Navazio *et al*. 2007). The heat stable Pi-CWE also promotes the growth of wild-type (WT) seedlings but not of seedlings of *P. indica*-insensitive mutants (Vadassery *et al*. 2009a). The extract and the fungus induce similar sets of genes in Arabidopsis roots, which also include genes with Ca^{2+} signaling-related functions (Vadassery *et al*. 2009a). The CWE induces $[Ca^{2+}]_{cyt}$ elevation preferentially in the roots and marginally in the shoots which is consistent with the fact that the endophyte is a root-colonizing fungus. The fungus and its CWE do not induce H_2O_2 production, an oxidative burst, programmed cell death and hypersensitive reactions in Arabidopsis root eventhough they activate MAPK3 and MAPK6 (Vadassery *et al*. 2009a). The active component in Pi-CWE which induces $[Ca^{2+}]_{cyt}$ elevation and growth promotion is not yet identified.

1.5.2 Role of Ca^{2+} signaling in pathogenic interactions and plant defense

Changes in $[Ca^{2+}]_{cyt}$ are also an essential early step in perception of pathogen and its PAMPs, and elicitation of subsequent innate immune response in plant cells to activate the plant's surveillance system against attempted pathogen invasion (Blume *et al*. 2000; Klüsener *et al*. 2002; Lecourieux *et al*. 2006). Cryptogein, β-1,3-glucan, Pep-13, Pep-25, INF1 and boehmerin from oomycete fungi; BcPG1 and Avr5 from necrotrophic fungi; and flg22, EF-Tu, lipopolysaccharides and harpin from bacteria are well known PAMPs which induce $[Ca^{2+}]_{cyt}$ elevation (cf. Lecourieux *et al*. 2006). It is reported that $[Ca^{2+}]_{cyt}$ elevation alters production

of ROS, activation of MAPK, the biosynthesis of stress hormones, production of antimicrobial compounds and the enhanced expression of defence genes (Lecourieux *et al*. 2006). Only specialized microbes are able to grow and reproduce on a given host plant, thereby being pathogenic, despite the activation of PAMP triggered defences (Jones and Dangl 2006). A prolonged $[Ca^{2+}]_{cyt}$ elevation appears to be correlated with phytoalexin accumulation in soybean or parsley cells (Mithöfer *et al*. 1999; Blume *et al*. 2000), and the establishment of the hypersensitive reaction (HR) in cowpea and tobacco (Xu and Heath 1998; Lecourieux *et al.* 2002). A large number of Ca^{2+} sensors including calcium-dependent protein kinases (CDPKs), calcium-calmodulin (CaM), calcineurin B-like (CBL) and Ca^{2+}-regulated protein kinases which decode Ca^{2+}-signatures, are also involved in plant defence against fungal pathogens and their PAMPs (Cheng *et al*. 2002; Lee and Rudd 2002; Yang and Poovaiah 2002; Takahashi *et al*. 2011). Incision of mildew resistance locus o (MLO), a transmembrane CaM-binding protein in barley, leads to powdery mildew resistance indicating that MLO has a negative regulatory function in plant defence and cell death (Kim *et al*. 2002). Thus calcium seems to play varied roles on pathogenic fungi perception acting as on-off switch for defense mechanism.

1.6 ROS in pathogenic and beneficial plant-fungus interactions

Phytopathogens and their PAMPs induce ROS and H_2O_2 production in plant cells as an early defense responses (Hu *et al*. 2009; O'Brien *et al*. 2012) whereas beneficial MAMP from AMF and Pi-CWE did not induce H_2O_2 production (Navazio *et al*. 2007; Vadassery *et al*. 2009a). ROS act as signaling molecules modulating several molecular and physiological processes in plants, but at higher concentration they are cytotoxic and making the cells more susceptible to the necrotrophic pathogens (Mittler *et al*. 2011). ROS play an important role in recognition of pathogen infection and activation of basal defense (c.f. Torres 2010; Heller and Tudzynski 2011). The necrotrophic fungi e.g. *Alternaria brassisicola, A. alternata* f. sp. *lycopersici* and *Botrytis cinerea* use these basal defense to facilitate their infection and successive colonization in their hosts by producing effectors/toxins (Gechev *et al*. 2004; Su'udi *et al*. 2011; Zhao *et al*. 2013). But the activation of ROS/H_2O_2 in biotrophic or hemibiotrophic interactions restricts subsequent growth and successive colonization of fungi (Lenz *et al*. 2011). Therefore, ROS/H_2O_2 induced during different plant-fungal interactions has different biological functions. Both Ca^{2+} fluxes and protein phosphorylation are required for the controlled generation of H_2O_2 (Neill *et al*. 2002). $[Ca^{2+}]_{cyt}$ elevations is reported upstream of

ROS production (Kawano and Muto 2000) and also as downstream of ROS production (Blume *et al*. 2000), indicating complex spatiotemporal Ca^{2+} elevation mechanisms. Membrane-bound NADPH oxidases are involved in ROS production. Interestingly, the AtRbohD and AtRbohF (*Arabidopsis* respiratory burst oxidase homologue)-NADPH oxidases have a dual function of mediating pathogen-associated ROS production (Torres *et al*. 2002). Furthermore, Rboh proteins have hydrophilic N-terminal regions containing two EF-hand motifs, suggesting that their activation is dependent on Ca^{2+}. Ogasawara *et al*. (2008) have shown that ROS production by AtRbohD was induced by ionomycin, a Ca^{2+} ionophore that induces Ca^{2+} influx into the cell. *P. indica* and its CWE did not induce H_2O_2 in Arabidopsis and rather they repressed it by activating different ROS scavenging enzymes (Vadassery *et al*. 2009a, b). In pathogenic interactions, transient $[Ca^{2+}]_{cyt}$ elevations induce defense gene activation, and stimulate an oxidative burst (Lecourieux *et al*. 2006), whereas in the *P. indica*-Arabidopsis interaction, defense gene activation is much lower, and H_2O_2 is not induced and accumulated (Vadassery *et al*. 2009a).

1.7 Redox responsive transcription factor1 (RRTF1)

ROS with signaling functions include H_2O_2, singlet oxygen ($^1O_2$), hydroxyl radical (OH^-) and superoxide anion radical (O_2^{-}) (Mehterov *et al*. 2012). Among the ROS marker genes, *RRTF1* is the most important one which is rapidly and transiently induced in response to abiotic and biotic stress resulting in an enhanced ROS/H_2O_2 formation in Arabidopsis (Toufighi *et al*. 2005; Khandelwal *et al*. 2008; Mehterov *et al*. 2012; Kerchev *et al*. 2013). *RRTF1* is an APETALA2/ethylene response transcription factor (TF) (AP2/ERF) and its expression in the nucleus is controlled by signals from the plastids (Khandelwal *et al*. 2008). Different ROS-inducing biotic stress like necrotrophic fungi (Heller and Tudzynski 2011) and PAMPs such as flg22 (flagellin 22) and chitin (Nürnberger *et al*. 1994) stimulate *RRTF1* expression whereas biotrophic powdery mildew fungus represses its expression (Pandey *et al*. 2010). RRTF1 is a component of a core redox signaling network that includes AP2, Myb, Zinc finger type, EDS1 (enhanced disease susceptibility1) and WRKY33 TFs (Khandelwal *et al*. 2008). *RRTF1* acts as a main hub to amplify ROS production in response to biotic stress and is upstream of oxidative burst (Mehterov *et al*. 2012; Kerchev *et al*. 2013).

1.8 *Alternaria brassicae* - A host specific necrotrophic pathogenic fungus

Necrotrophic pathogenic fungi are major class of phytopathogens causing serious diseases in

agriculturally important crop plants resulting in heavy crop loss. *Alternaria brassicae* (Berk.) Sacc., is one of the necrotrophic deuteromycete fungi (Figures 5A-B) causing black/grey leaf spot disease in members of brassicaceae family including *A. thaliana* (Figures 5C-D). It is a seed-, air- and soil-borne fungus distributed throughout the world in an endemic form and causes lesions with grayish, brownish, or blackish centers and chlorotic margins on the leaves, stems, siliques and even on roots (Bains and Tewari 1987), ultimately results in the complete death of the plant. Under favourable environmental conditions, the fungus devastates the crop within a shorter period. The fungus manifests the symptoms, and causes the disease mostly by producing host-specific toxins (HSTs) which are toxic only to the host plants, and are primary determinant of virulence or pathogenicity (Walton 1996; Moebius and Hertweck 2009; Pedras and Khallaf 2012). Destruxin B and sirodesmin PL are two phytotoxins produced by *A. brassicae* (Pedras and Khallaf, 2012) which elicit a number of phytoalexins including camalexin in crucifers (Pedras and Khallaf 2012). Little is known about the early signaling events and molecular mechanisms involved in the pathogenic interaction of Arabidopsis and *A. brassicae*.

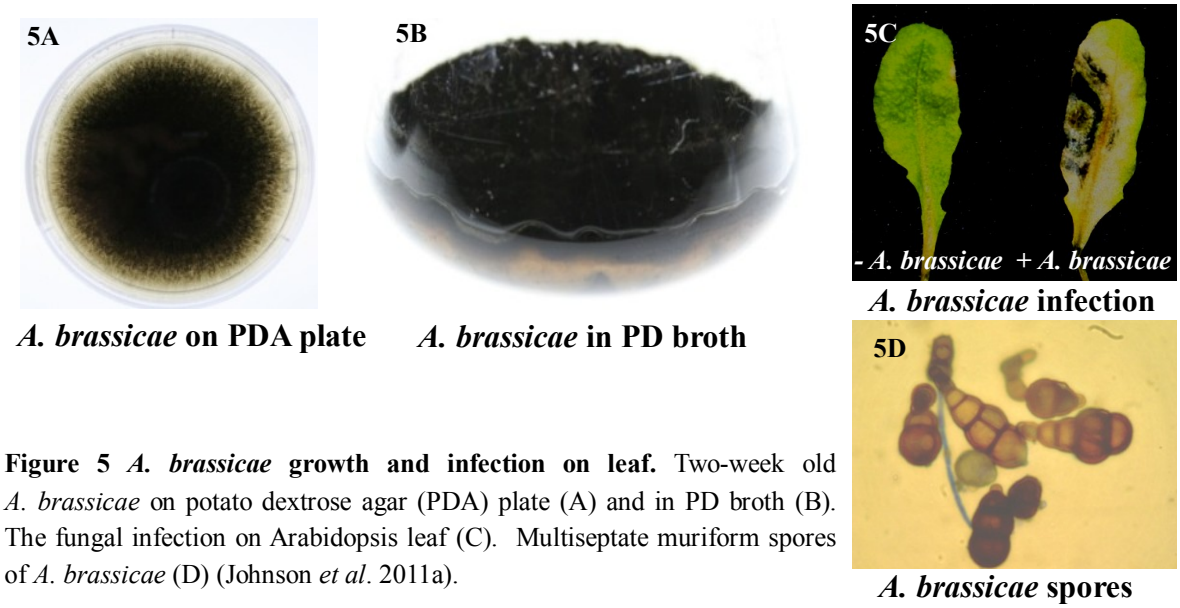


Figure 5 *A. brassicae* growth and infection on leaf. Two-week old *A. brassicae* on potato dextrose agar (PDA) plate (A) and in PD broth (B). The fungal infection on Arabidopsis leaf (C). Multiseptate muriform spores of *A. brassicae* (D) (Johnson *et al*. 2011a).

In my study, I have used both beneficial interaction (*A. thaliana-P. indica*) and pathogenic interaction (*A. thaliana-A. brassicae*) and their bMAMPs/PAMPs preparations to elucidate the role of $[Ca^{2+}]_{cyt}$ in plant-microbe interactions using different mutants which lacks $[Ca^{2+}]_{cyt}$ elevation and ROS amplification.

2. Objectives

The aim of this thesis is to elucidate the role of $[Ca^{2+}]_{cyt}$ signaling in the beneficial interaction between *A. thaliana* and *P. indica*, and the pathogenic interaction between *A. thaliana* and the necrotroph, *A. brassicae*.

To fulfil the above aim, the following objectives were addressed.

- to identify and purify the component(s) from *P. indica*, which induce(s) $[Ca^{2+}]_{cyt}$ elevation and promote(s) growth in *A. thaliana*,
- to isolate Arabidopsis mutants which do not respond to *P. indica* factor-induced $[Ca^{2+}]_{cyt}$ elevation,
- to elucidate the role of $[Ca^{2+}]_{cyt}$ signaling in the beneficial (*A. thaliana-P. indica*) and pathogenic (*A. thaliana-A. brassicae*) interactions,
- to investigate the role of REDOX RESPONSIVE TRANSCRIPTION FACTOR1 (RRTF1) in the above beneficial and pathogenic interactions in *A. thaliana*,
- to study the role of auxin in the *A. thaliana-P. indica* interaction,

3. Manuscript overview

3.1 Manuscript 1

Growth promotion of Chinese cabbage and Arabidopsis by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin

Yin-Chen Lee*, Joy Michal Johnson*, Ching-Te Chien, Chao Sun, Daguang Cai, Binggan Lou, Ralf Oelmüller and Kai-Wun Yeh**
Molecular Plant Microbe Interactions (2011), 24(4): 421-431.
(*) contributed equally
(**) corresponding author; email: ykwbppp@ntu.edu.tw

This publication describes how auxin is differentially involved in the *P. indica*-mediated growth promotion in Chinese cabbage (*Brassica campestris* subsp. *chinensis*) and *Arabidopsis thaliana* seedlings. We could demonstrate that the growth promotion in Chinese cabbage is mediated by the modulation of auxin signal and transport carrier proteins which increases auxin levels; whereas these proteins are not involved in the fungus induced growth promotion in *A. thaliana* as the auxin levels are not affected in the colonized *A. thaliana*. Mutants impaired in auxin synthesis, signaling and transportation (*aux1*, *aux1/axr4* and *rhd6*) responded well to *P. indica* in Arabidopsis. This paper could further show that component(s) from *P. indica* but not auxin stimulated growth of Chinese cabbage and Arabidopsis seedlings.

All experiments were designed by K.W.Y. and Y.C.L. on Chinese cabbage and by R. O. and J.M.J. on Arabidopsis. Y.C.L. performed the experiments on Chinese cabbage and J.M.J. on Arabidopsis. The overexpression of Bcaux1 in Arabidopsis was done by Y.C.L and C.T.C. J.M.J. partially purified Pi-CWE and Pi-WDE. J.M.J. and C.S. did growth promotion assay. D.C. and B.L. contributed to the discussion. R.O. and K.W.Y. wrote the paper.

3.2 Manuscript II

Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*

Pyniarlang L. Nongbri*, Joy Michal Johnson*, Irena Sherameti, Erich Glawischnig, Barbara Ann Halkier and Ralf Oelmüller**

Molecular Plant Microbe Interactions (2012), 25(9): 1186-1197.

(*) contributed equally

(**) corresponding author; email: b7oera@hotmail.de

This publication explores the role of indole glucosinolate and camalexin in the beneficial interaction between *A. thaliana* and *P. indica*. The double knock out *cyp79B2 cyp79B3* mutant which is devoid of indole glucosinolate and camalexin, and *pad3* defective in camalexin biosynthesis did not show growth promotion under short- and long-term cocultivation experiments with *P. indica* due to the over-colonization of the fungus which shift the interaction from mutualism to parasitism. This paper further shows $[Ca^{2+}]_{cyt}$ signaling is essential for the indole glucosinolate and camalexin biosynthesis. We propose that constitutive levels of glucosinolate and camalexin are important to restrict degree of fungal colonization to a mutually beneficial level for both the partners.

All the experiments are designed by R. O; P. L. N. investigated the knock out *cyp79B2 cyp79B3* and *pad3* mutants. J. M. J. analyzed the role of $[Ca^{2+}]_{cyt}$ signaling in *P. indica-A. thaliana* interaction using mutants. E. G. measured camalexin levels. B. A. H. created the *cyp79B2 cyp79B3* double mutant. I. S. coordinated the work.

3.3 Manuscript III

Agony to Harmony - What decides? Calcium signaling in beneficial and pathogenic plant-fungus interactions - What can we learn from the *Arabidopsis/Piriformospora indica* symbiosis?

Joy Michal Johnson and Ralf Oelmüller*

In *Molecular Microbial Ecology of the Rhizosphere (2013), Volume 2, pp 833-850,* First Edition. Edited by Frans J. de Bruijn.

(*) corresponding author; email: b7oera@hotmail.de

This book chapter reviews the role of $[Ca^{2+}]_{cyt}$ signaling in beneficial and pathogenic fungal interactions. We attempted to summarise the origin and function of $[Ca^{2+}]_{cyt}$ signaling, biotic and abiotic stimuli inducing $[Ca^{2+}]_{cyt}$ elevation, measurement of $[Ca^{2+}]_{cyt}$ elevation and the involvement of $[Ca^{2+}]_{cyt}$ in different stages of pathogenesis and endosymbosis in plant cells. The importance of $[Ca^{2+}]_{cyt}$ signaling in Arbuscular Mycorrhiza and *P. indica* endosymbiosis is reviewed in details.

J.M.J. and R.O. wrote this book chapter.

3.4 Manuscript IV (in preparation)

Growth and Defense - Ca^{2+} signaling in unstable continuum: *Piriformopsora indica*-derived trisaccharide induces intracellular calcium elevation, promotes growth and confers resistance in *Arabidopsis thaliana*

Joy Michal Johnson, Ravikumar Maddula, Michael Reichelt, Sybille Lorenz, Mitsuhiro Matsuo, Rinesh Godfrey, Jyothilakshmi Vadassery, Pyniarlang L. Nongbri, Frank D-Böhmer, Jonathan Gershenzon, Bernd Schneider, Ales Svatos and Ralf Oelmüller*
(*) corresponding author; email: b7oera@hotmail.de

In this work, we report that a trisaccharide (*m/z* = 505.1748; Pi-504) is the active component in the CWE of *P. indica* which induces $[Ca^{2+}]_{cyt}$ elevation in roots and also promotes growth in *A. thaliana*. Further we have screened for and identified mutants which do not respond to Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation. The mutants are impaired in *P. indica*-induced growth promotion and are highly susceptible to *Alternaria brassicae* infection, sensitive to *A. brassicae*-toxin preparation, and oxidative and salt stress. *A. brassicae*-toxin-induced Ca^{2+} signaling cascades which lead to hypersusceptibility of Arabidopsis to *A. brassicae* are repressed by Pi-504-induced Ca^{2+} signaling cascades. We could also prove that loss-of-function of gene involved in sensing Pi-504 and inducing $[Ca^{2+}]_{cyt}$ elevation, also affected the tight regulation of reactive oxygen species (ROS) production which is downstream of $[Ca^{2+}]_{cyt}$ elevation. These results suggest that the *P. indica*-induced $[Ca^{2+}]_{cyt}$ elevation is crucial for the growth promotion, and the activation of defense against different abiotic and biotic stress.

J.M.J. and R.O. designed and planned the research. J.M.J. did experiments. J.M.J., R.M., S.L., M.R., B.S. and AS purified and analysed Pi-504. J.M.J. and R.G. performed ROS experiments. M.M. developed the overexpressors. J.M.J., P.L.N. and J.V. analysed data. J.V., F.D.B. and J.G. contributed to the discussion. J.M.J. and R.O. wrote the article. R.O. supervised the work.

3.5 Manuscript V (under revision, The Plant Cell)

REDOX RESPONSIVE TRANSCRIPTION FACTOR1 amplifies the formation of reactive oxygen species in *Arabidopsis thaliana* shoots and roots

Mitsuhiro Matsuo*, Joy Michal Johnson*, Rinesh Godfrey, Junichi Obokata, Frank-D. Böhmer and Ralf Oelmüller**
(*) even contribution
(**) corresponding author; email: b7oera@hotmail.de

In this paper, we showed the role of a highly conserved *REDOX RESPONSIVE TRANSCRIPTION FACTOR1* (*RRTF1*) in amplification of reactive oxygen species (ROS) in *A. thaliana* under abiotic and biotic stress conditions. The differential responses of *RRTF1* in beneficial and pathogenic interactions in *A. thaliana* were also clearly demonstrated. Loss-of-function of *RRTF1* restricts and overexpression promotes ROS production in response to biotic and abiotic stress. We could further show that the beneficial root endophyte *P. indica* represses ROS accumulation locally in roots and systemically in shoots by repressing RRTF1 and activating scavenging enzymes. The microarray data shows that RRTF1 is a main hub for the amplification of ROS by activating different ROS/H_2O_2 generating/marker genes in *A. thaliana*.

R.O., J.M.J., and M.M. designed the research. M.M. developed over expressers. J.M.J. and M.M. did microarray experiments. J.M.J. did all experiments with fungi and light stress. J.M.J. and R.G. performed ROS measurements. J.M.J., M.M., and R.O. analysed data. F.D.B., and J.O. contributed to the discussion and article. R.O. planned and supervised the research. R.O., J.M.J., and M.M. wrote the article.

3.6 Manuscript VI (in preparation)

A cell wall extract and exudates from *Alternaria brassicae* induce intracellular calcium elevation and is crucial for the enhanced tolerance to biotic and abiotic stress in *Arabidopsis thaliana*

Joy Michal Johnson, Michael Reichelt, Pyniarlang L. Nongbri, Jyothilakshmi Vadassery, Jonathan Gershenzon and Ralf Oelmüller*
(*) corresponding author; email: b7oera@hotmail.de

In this paper, we describe how a cell wall extract (CWE), exudates from germinating spores and mycelium, and a toxin preparation from the pathogenic fungus, *A. brassicae* induce receptor mediated $[Ca^{2+}]_{cyt}$ elevation in roots. Mutants, which do not respond to CWE and exudates in $[Ca^{2+}]_{cyt}$ elevation, responded well to toxin preparation, were screened and identified. We could further show that the CWE- and exudates-induced $Ca^{2+}]_{cyt}$ elevation is involved in the activation of basal defense, and thereby in enhanced tolerance to abiotic and biotic stress; whereas toxin-induced $Ca^{2+}]_{cyt}$ elevation is involved in hypersusceptibility to the fungus.

J.M.J. and R.O. designed and planned the research. J.M.J did all experiments. J.M.J. and M.R. performed phytohormones, glucosinolates and camalexin measurements. J.M.J. and P.L.N. analysed data. P.L.N., J.V. and J.G. contributed to the discussion and article. J.M.J. and R.O. wrote the article. R.O. supervised the research.

3.7 List of other publications (Kept as supplementary publications in CD)

1. Balancing defense and growth - Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*.
Pyniarlang L. Nongbri, Khabat Vahabi, Anna Mrozinska, Eileen Seebald, Chao Sun, Irena Sherameti, Joy M. Johnson and Ralf Oelmüller.
Symbiosis (2012), 58: 17-28.

2. Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation - A model system to study plant beneficial traits.
Joy Michal Johnson, Irena Sherameti, Anatoli Ludwig, Pyniarlang L. Nongbri, Chao Sun, Binggan Lou, Ajit Varma and Ralf Oelmüller.
Endocytobiosis and Cell Research (2011), 21: 101-113.

3. Calcium signaling and cytosolic calcium measurements in plants.
Joy Michal Johnson, Pyniarlang Nongbri, Irena Sherameti and Ralf Oelmüller.
Endocytobiosis and Cell Research (2011), 21: 64-76.

4. Fungal staining tools to study the interaction between the beneficial endophyte *Piriformospora indica* with *Arabidopsis thaliana* roots.
Khabat Vahabi, Joy Michal Johnson, Corinna Drzewiecki and Ralf Oelmüller.
Endocytobiosis and Cell Research (2011), 21: 77-88.

5. *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid localized CAS protein.
Chao Sun, Joy M. Johnson, Daguang Cai, Irena Sherameti, Ralf Oelmüller and Binggan Lou.
Journal of Plant Physiology (2010), 167: 1009-1017.

6. The central role of iron and calcium for plant/microbe interaction and shaping microbial communities in the soil.
Sherameti Irena, Joy Michal Johnson, Nongbri Pyniarlang and Oelmüller Ralf.
Albanian Journal of Agricultural Sciences (2010), 9(3): 1-24.

7. Mutualism or parasitism: life in an unstable continuum. What can we learn from the mutualistic interaction between *Piriformospora indica* and *Arabidopsis thaliana*?

Joy Michal Johnson and Ralf Oelmüller.

Endocytobiosis and Cell Research (2009), 19: 81-110.

Book Chapters

8. *Piriformospora indica* promotes growth of chinese cabbage by manipulating auxin homeostasis - Role of auxin in symbioses.

Joy Michal Johnson, Yin-Chen Lee, Iris Camehl, Chao Sun, Kai-Wun Yeh and Ralf Oelmüller.

In. *Piriformospora indica - Sebacinales and their biotechnological applications (2013), Soil Biology 33: 139-148.* A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg. Germany.

9. Standardized conditions to study beneficial and nonbeneficial traits in the *Piriformospora indica/Arabidopsis thaliana* interaction.

Joy Michal Johnson, Irena Sherameti, Pyniarlang L. Nongbri, and Ralf Oelmüller.

In. *Piriformospora indica - Sebacinales and their biotechnological applications (2013), Soil Biology 33: 325-343.* A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg. Germany.

10. Role of defense compounds in the beneficial interaction between *Arabidopsis thaliana* and *Piriformospora indica*.

Iris Camehl, Irena Sherameti, Eileen Seebald, Joy M. Johnson, and Ralf Oelmüller.

In. *Piriformospora indica - Sebacinales and their biotechnological applications (2013), Soil Biology 33: 239-250.* A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg. Germany.

11. The Symbiotic Fungus *Piriformospora indica*: Review.

Varma A., Sherameti I., Tripathi S., Prasad R., Das A., Sharma M., Bakshi, M., Johnson J.M., Bhardwaj S., Arora M., Rastogi K., Agarwal A., Kharkwal A.C., Talukdar S., Bagde U.S., Bisaria V.S., Upadhyaya C.P., Won P.S., Chen Y., Ma J., Lou B., Adya A., Zhong L., Meghvanshi M.K., Gosal S.K., Srivastava R.B., Johri A.K., Cruz C. and Oelmüller R. In. *Fungal Associations, 2nd Edition (2012), The Mycota IX: 231-254.* B. Hock (Ed.). © Springer-Verlag Berlin Heidelberg. Germany.

4.1 Manuscript 1

Growth promotion of Chinese cabbage and Arabidopsis by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin

Yin-Chen Lee*, Joy Michal Johnson*, Ching-Te Chien, Chao Sun, Daguang Cai, Binggan Lou, Ralf Oelmüller and Kai-Wun Yeh

Molecular Plant Microbe Interactions. (2011), 24(4): 421-431.
(*) contributed equally

MPMI Vol. 24, No. 4, 2011, pp. 421–431. doi:10.1094/MPMI-05-10-0110. © 2011 The American Phytopathological Society

e-Xtra*

Growth Promotion of Chinese Cabbage and *Arabidopsis* by *Piriformospora indica* Is Not Stimulated by Mycelium-Synthesized Auxin

Yin-Chen Lee,[1] Joy Michal Johnson,[2] Ching-Te Chien,[3] Chao Sun,[2] Daguang Cai,[4] Binggan Lou,[5] Ralf Oelmüller,[2] and Kai-Wun Yeh[1]

[1]Institute of Plant Biology, College of Life Science, National Taiwan University, Taipei, 106, Taiwan; [2]Friedrich-Schiller-Universität Jena, Institut für Allgemeine Botanik und Pflanzenphysiologie, Dornburger Str.159, 07743 Jena, Germany; [3]Taiwan Forestry Research Institute, Council of Agriculture, Nan-Hai Road, 53, Taipei, 106, Taiwan; [4]Molecular Phytopathology Institute, University of Kiel, Kiel, Germany; [5]Institute of Biotechnology, Zhejiang University, Hangzhou 310029, China

Submitted 12 May 2010. Accepted 14 December 2010.

Piriformospora indica*, an endophytic fungus of the order Sebacinales, interacts with the roots of a large variety of plant species. We compared the interaction of this fungus with Chinese cabbage (*Brassica campestris* subsp. *chinensis*) and *Arabidopsis* seedlings. The development of shoots and roots of Chinese cabbage seedlings was strongly promoted by *P. indica* and the fresh weight of the seedlings increased approximately twofold. The strong stimulation of root hair development resulted in a bushy root phenotype. The auxin level in the infected Chinese cabbage roots was twofold higher compared with the uncolonized controls. Three classes of auxin-related genes, which were upregulated by *P. indica* in Chinese cabbage roots, were isolated from a double-subtractive expressed sequence tag library: genes for proteins related to cell wall acidification, intercellular auxin transport carrier proteins such as AUX1, and auxin signal proteins. Overexpression of *B. campestris BcAUX1* in *Arabidopsis* strongly promoted growth and biomass production of *Arabidopsis* seedlings and plants; the roots were highly branched but not bushy when compared with colonized Chinese cabbage roots. This suggests that *BcAUX1* is a target of *P. indica* in Chinese cabbage. *P. indica* also promoted growth of *Arabidopsis* seedlings but the auxin levels were not higher and auxin genes were not upregulated, implying that auxin signaling is a more important target of *P. indica* in Chinese cabbage than in *Arabidopsis*. The fungus also stimulated growth of *Arabidopsis aux1* and *aux1/axr4* and *rhd6* seedlings. Furthermore, a component in an exudate fraction from *P. indica* but not auxin stimulated growth of Chinese cabbage and *Arabidopsis* seedlings. We propose that activation of auxin biosynthesis and signaling in the roots might be the cause for the *P. indica*-mediated growth phenotype in Chinese cabbage.

Plants are exposed to a large variety of microorganisms in the rhizosphere, which can be beneficial, neutral, or pathogenic for the host. Beneficial microbes often stimulate plant growth and biomass production, which result from a better nutrient exchange between the two symbionts and the activation of signaling pathways controlling plant development and performance (Morgan et al. 2005). The direct target of soilborne microorganisms is the plant root system, for which auxin is a crucial growth regulator. Auxins stimulate lateral root growth and root hair development (Fukaki and Tasaka 2009), similar to many beneficial microbes (Gupta et al. 2000; Montesinos et al. 2002; Morgan et al. 2005). Inhibition of auxin transport reduces root branching (Casimiro et al. 2001; Reed et al. 1998) and prevents the effect of beneficial microbes. In *Arabidopsis*, a number of auxin-resistant mutants and auxin overproducers with altered root and root hair development have been identified. Mutants *axr1* and *axr2* show a reduced number of lateral roots (Estelle and Somerville 1987), whereas *rooty* mutants such as *alf1* or *superroot1* (*sur1*) with elevated auxin levels show an increased development of lateral roots (Boerjan et al. 1995; Celenza et al. 1995; King et al. 1995). Furthermore, the *aux1* mutant is impaired in auxin influx into the cell and auxin acropetal and basipetal transport in the root tip (Swarup et al. 2001). AXR4 is required for proper trafficking and location of AUX1 via the lateral root cap to the elongating epidermal cells in the roots (Dharamasiri et al. 2006). Other auxin mutants are impaired in the correct localization of auxin transporters (such as *doc1/big*) (Gil et al. 2001), in the basipetal auxin transport (*eir1/pin2*) (Luschnig et al. 1998), or in an efflux carrier (*pin1* [Feraru and Friml 2008; Paponov et al. 2005] and *pin2*, *pin3* [Friml 2003]). PIN3 is localized symmetrically in columella cells and mediates lateral auxin distribution to all sides of the root cap. After the root is turned by 90 degrees, PIN3 rapidly relocates to the bottom side of columella cells and, thus, probably regulates auxin flux to the lower side of the root (Friml 2003). Auxin is further transported through lateral root cap and epidermis cells basipetally by a PIN2-dependent route. This basipetal transport also requires AUX1-dependent auxin influx into the cell. AUX1 is present in the same cells as PIN3 and PIN2 (Friml 2003). Root biomass production can be stimulated by the promotion of longitudinal growth of the primary and lateral roots or the initiation of additional lateral root primordia. RHD6 is required for root hair initiation and the *rhd6* mutation can be rescued by auxin (Masucci and Schiefelbein 1994) or auxin-producing microbes. Auxin-induced increase in root biomass can be monitored by the stimulation of auxin-re-

Y.-C. Lee and J. M. Johnson contributed equally to this study.

Corresponding author: K.-W. Yeh; Telephone: +886-2-33662536; Fax: +886-2-23622703; E-mail: ykwbppp@ntu.edu.tw

*The *e*-Xtra logo stands for "electronic extra" and indicates that a supplementary figure is published online and Figs. 5 and 6 appear in color online.

sponsive promoters fused to reporter genes. The best-characterized examples are transgenic *Arabidopsis* lines harboring *DR5::uidA* (Ulmasov et al. 1995, 1997) or *BA3::uidA* (Oono et al. 1998) constructs.

Little is known about the molecular basis of root growth promotion by beneficial microbes or fungi (Pieterse and Dicke 2007). It is obvious that microbe-induced stimulation of root growth or changes in root architecture involve auxins or interferes with the auxin metabolism or signaling in the roots (Contreras-Cornejo et al. 2009; Schäfer et al. 2009; Sirrenberg et al. 2007; Vadassery et al. 2008). Microbes often release plant-growth-regulating compounds, including auxin, into the medium or the rhizosphere. More recently, it became obvious that auxin also plays an important role in plant defense (Kazan and Manners 2009). These processes can occur in many ways: by elevating auxin synthesis, releasing auxin from stores or conjugates, stimulating its transport, or activating auxin-induced genes in various tissues which are required for cell growth or proliferation (Ludwig-Müller 1999, 2000; Ludwig-Müller et al. 2005). The auxin-induced signaling pathway involves the F-box protein TIR1 receptor. Its activation leads to ubiquitination-based degradation of transcriptional repressors and complex transcriptional reprogramming (Vanneste and Friml 2009). In addition, several examples demonstrate that auxin or auxin-related compounds can be synthesized by the microbe which can trigger the plant auxin signaling pathway (Contreras-Cornejo et al. 2009). Like exogenous application of auxin, beneficial microbes can suppress the root hair formation defects of *RHD6* by synthesizing auxin-related compounds (Contreras-Cornejo et al. 2009).

We studied the molecular mechanisms by which the endophyte *Piriformospora indica* promotes growth and biomass production of various plant species. *P. indica* belongs to the new order of Sebacinales and can be easily grown on various complex and minimal substrates even without a host plant (Oelmüller et al. 2004, 2009; Sahay and Varma 1999). Because it colonizes the roots of many plant species, including mono- and dicots, mosses, and all tested crop plants, we propose that the fungus should target general growth-promoting programs in the plants. Recently, we challenged the fungus with a major crop in Asia, *Brassica campestris*, the Chinese cabbage. We confirmed the beneficial effects of *P. indica* on growth and biomass production (Sun et al. 2010). The comparative analysis of *P. indica* on Chinese cabbage and *Arabidopsis* growth revealed that auxin synthesis and signaling is a more important target of the fungus in Chinese cabbage than it is in *Arabidopsis*.

RESULTS

Infection of Chinese cabbage by *P. indica* promotes growth and stimulates root hair development.

Seedlings of Chinese cabbage (*B. campestris* subsp. *Chinensis*) and *Arabidopsis* (as control), aseptically germinated on 1/2 Murashige-Skoog (MS) medium (Murashige and Skoog 1962) for 5 days, were transferred to a fresh medium and co-cultivated with *P. indica* by depositing mycelial discs (or agar discs for control plants) next to the root tissue (Fig. 1A, upper panel, for Chinese cabbage). Seven days after infection, numerous lateral roots were visible on the Chinese cabbage roots. We also observed a strong increase in root hair development, resulting in a bushy phenotype (Fig. 1A, lower panel). Both the length and the number of the lateral roots and root hairs increased in the presence of the fungus (Figs. 1 and 2). After 7 days of co-cultivation, the size and fresh weight of the infected Chinese cabbage roots (and shoots; data not shown) was at least twofold higher compared with the uninfected controls. An increase of the number of fungal plaques per seedling caused only a marginal additional effect (Fig. 2). Growth promotion extended to adult plants in pots (Fig. 1B). Microscopic analysis confirmed that the mycelium not only grew around the root system but also penetrated into the intracellular space of the Chinese cabbage root tissue (Fig. 1C). In several root cells, arthrospores of *P. indica* were visible. These results suggest that the changes in root morphology and the increase in biomass are caused by efficient root colonization. Quite similar effects have previously been reported for *Arabidopsis* seedlings (Peškan-Berghöfer et al. 2004). However, comparison of the two species demonstrated that root hair development was much more strongly stimulated in colonized Chinese cabbage than in *Arabidopsis*. We never observed a bushy root phenotype in *Arabidopsis* (Figs. 1D and 2). Furthermore, the increase of the fresh weight of *Arabidopsis* seedlings (approximately 50%) after 7 days of co-cultivation with *P. indica* was less than half of the increase observed for Chinese cabbage, and the growth response of *Arabidopsis* seedlings was less dependent on the number of fungal plaques which were applied to the roots (Fig. 2).

Auxin levels were determined for root and leaf tissues of Chinese cabbage and *Arabidopsis* seedlings, co-cultivated separately or together in petri dishes with and without *P. indica*. The auxin level of the infected Chinese cabbage roots was twofold higher compared with the uninfected controls, whereas no difference was observed for leaf tissues (Fig. 3). This suggests that the strong root branching, the bushy phenotype, and the increase in biomass of the infected Chinese cabbage seedlings are caused by higher auxin levels. In contrast, no significant difference could be detected for the auxin level in *Arabidopsis* roots and shoots, even if the two types of seedlings were grown in the same petri dish in the presence of *P. indica* (Fig: 3). This clearly demonstrates differences in the auxin levels in the colonized roots of the two species.

Isolation of *P. indica* target genes from a double-subtracted expressed sequence tag cDNA library.

In order to identify genes which are targeted by *P. indica* in Chinese cabbage roots, a subtractive expressed sequence tag (EST) cDNA library was constructed from cDNAs of infected plants as tester and cDNAs from both control root tissues and *P. indica* mycelium as drivers. The nucleotide sequences of 265 clones with an average length of 900 bp from the double-subtracted EST library were determined. Their annotation uncovered 193 protein-coding cDNAs. In all, 13 EST genes encoding auxin-related proteins were selected for this analysis. These proteins can be grouped into three categories (Table 1): (i) proteins involved in cell wall (apoplast) acidification, such as the H^+-ATPase; (ii) proteins involved in auxin transport, such as AUX1 and BiG; and (iii) proteins involved in auxin signal transduction, such as TIR/AFB (auxin receptor), AUX/indole-acetic acid (IAA) (auxin repressor), and E2-ubiquitin conjugating enzymes for auxin degradation (Table 1). Thus, auxin-related proteins might be involved in growth promotion and the establishment of the highly branched and bushy root architecture in colonized Chinese cabbage seedlings.

Characterization of auxin-related genes activated by *P. indica* in Chinese cabbage.

Northern blot analyses (Fig. 4A) and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Fig. 4B) confirmed that all isolated auxin-related genes were upregulated in *P. indica*-infected Chinese cabbage roots, although to different extents. First, upregulation of the *AUX1* and *BIG* mRNA levels implies that the fungal interaction results in a more efficient influx of auxin into the root cell. Because the *PIN3* mRNA level is slightly downregulated by the fungus, auxin release from the

cell could be reduced. Second, the higher mRNA levels for the vacuolar-type H^+-ATPase subunit B2 and the pyrophosphate-energized vacuolar membrane H^+ pump (Fig. 4A and B) in infected Chinese cabbage roots indicate that cell wall acidification is enhanced, as proposed for homologues of these two proton pumps in other plant species which participate in auxin-mediated growth promotion. Finally, crucial genes for auxin-signaling components are also upregulated in *P. indica*-infected Chinese cabbage. *TIR1* coding for an F-box and leucine-rich-repeat protein, an auxin receptor in the nucleus, is upregulated in response to the fungus (Fig. 4A and B). The F-box protein was first discovered as a component of the Skp1-cullin-F box protein (SCF) E3 ubiquitin ligase complex (Vanneste and Friml 2009). *IAA7* is a member of the auxin-responsive *AUX/IAA* family. AUX/IAA proteins are important negative regulators of auxin-regulated genes. They regulate gene transcription indirectly by binding to the DNA-associated ARF protein (Gray et al. 2001). *UBC10* codes for an E2 ubiquitin-conjugating enzyme, one of three enzymes in the ubiquitin-protein conjugation pathway that conjugates ubiquitin to the SCF complex. Another *P. indica*-responsive gene encodes the 26S proteasome subunit 4, which is involved in the degradation of AUX/IAA proteins (Fig. 4A and B). In total, the differential expression of these auxin-related genes demonstrates that they play a crucial role in *P. indica*-mediated growth promotion and alteration of root morphology in Chinese cabbage.

The *Arabidopsis* homologues of *P. indica*-responsive and auxin-related genes from Chinese cabbage are not upregulated in colonized *Arabidopsis* roots.

Because of the difference in the root hair phenotypes and auxin levels in *P. indica*-colonized Chinese cabbage and *Arabidopsis* roots, we tested whether the *Arabidopsis* homologues of the auxin-related genes identified in Chinese cabbage are up-regulated in colonized *Arabidopsis* roots. The experiments were performed with RNA isolated from *Arabidopsis* seedlings grown in a petri dish together with Chinese cabbage seedlings (Fig. 1D). None of the tested auxin-related genes was upregulated in

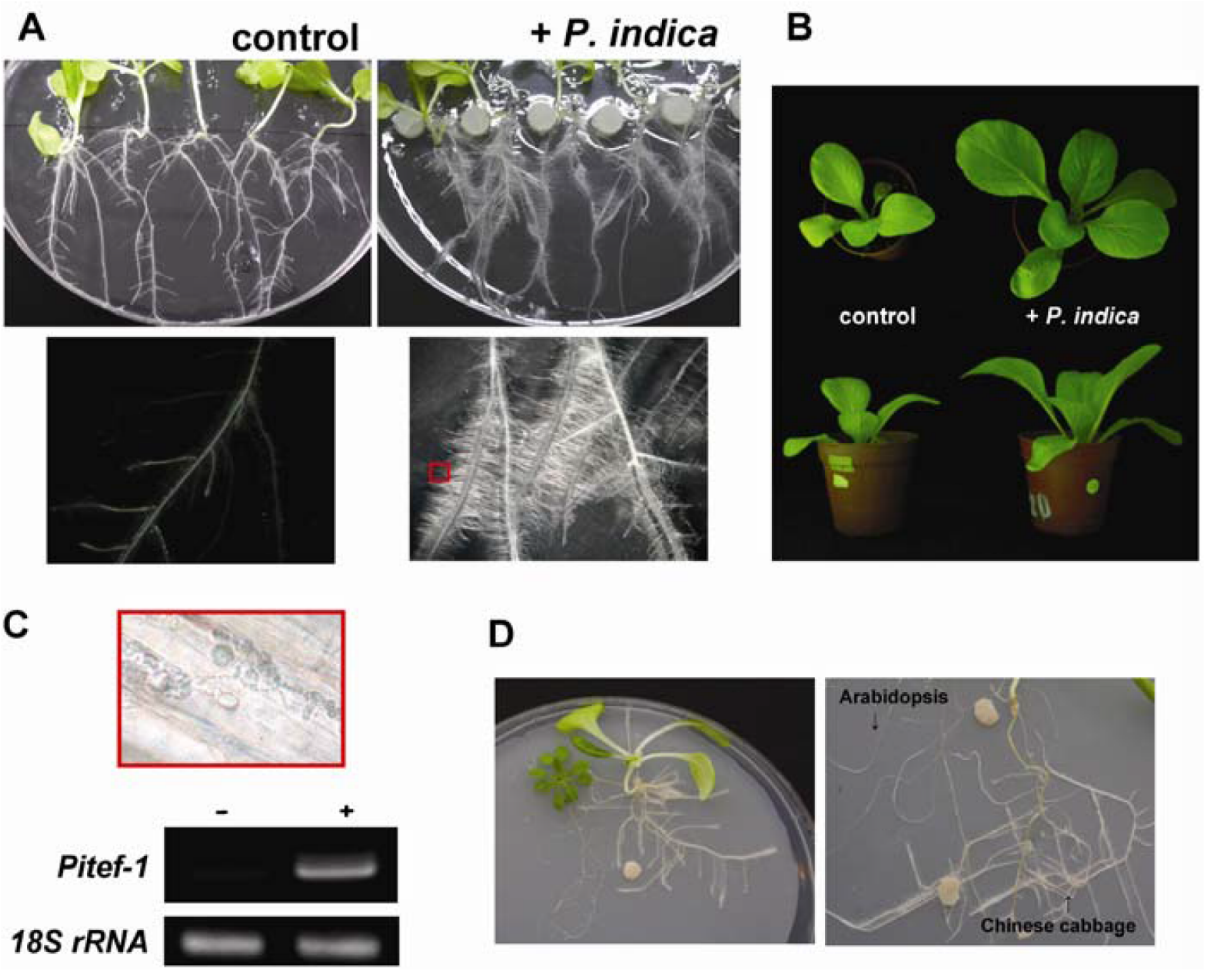


Fig. 1. Interaction of *Piriformospora indica* with Chinese cabbage. **A,** Branching and root hair phenotype of Chinese cabbage at 7 days after fungal co-cultivation with *P. indica*. Five days after seed germination, seedlings were co-cultivated with *P. indica* and grown on $^1/_2$ Murashige-Skoog medium for 7 days. Left panel: mock treatment; right panel: co-cultivated by *P. indica*. Lower panels enlarge the roots. For this picture, five seedlings and five fungal plaques were used. **B,** Biomass of Chinese cabbage promoted by *P. indica*. Plants co-cultivated with *P. indica* in petri dishes for 7 days (as in A) were moved to pots and further cultivated for an additional 30 days. **C,** Microscopic structures of roots of Chinese cabbage showing the hyphal penetration by *P. indica*. Top: dissected root tissue stained with lactophenol or cotton blue. Hyphae and arthrospores are observed. Bottom: molecular identification of the marker gene *Pitef-1* by polymerase chain reaction, indicating fungal colonization of the root tissue (Bütehorn et al. 2000). **D,** *Arabidopsis* and Chinese cabbage seedlings (left) and roots (right) co-cultivated with *P. indica* in the same petri dish. Notice the difference in the root architecture. One or two fungal plaques are visible.

Arabidopsis roots and many of them were even downregulated (Fig. 4B), although these results were not significantly different. This implies that auxin is less important for the beneficial interaction between *P. indica* and *Arabidopsis* than it is for the interaction with Chinese cabbage, although seedlings of both species were taller in the presence of the fungus.

Overexpression of BcAUX1 in *Arabidopsis* mimics the *P. indica* effects but does not induce root hair development.

AUX1 is a permease-like auxin influx carrier which functions in the short-distance cell-to-cell transport of the hormone and facilitates active auxin uptake into the cell (Raven 1975). Loss of AUX1 function results in growth inhibition and a reduced response to gravity (Maher and Martindale 1980). To confirm the importance of AUX1 in *P. indica*-mediated growth promotion and alteration in the root morphology in Chinese cabbage, a full-length *BcAUX1* cDNA was isolated by 5′ and 3′ rapid amplification of cDNA ends (RACE). A 1,650-bp-long cDNA fragment was obtained which contained a 1,479-bp-long open reading frame coding for a 493-amino-acid-long protein with a predicted molecular mass of 59 kDa (accession no. GU191828). This cDNA was subsequently delivered into *Arabidopsis* by *Agrobacterium tumefaciens*-mediated transformation. Transgenic T2 *Arabidopsis* seedlings and plants overexpressing *BcAUX1* under the control of 35S promoter were significantly bigger than the untransformed controls (Fig. 5). These results demonstrate that *BcAUX1* plays a key role in the response of Chinese cabbage to *P. indica* and heterologous expression of this gene induces similar growth responses in *Arabidopsis*, even in the absence of the fungus. However, although *P. indica* and overexpression of *BcAUX1* promote root growth and lateral root development in *Arabidopsis*, a bushy root hair phenotype was not detectable.

AUX1, AUX1/AXR4, and RHD6 seedlings respond to *P. indica*.

Chinese cabbage AUX1 is an important growth regulator in *Arabidopsis*, although the endogenous *AUX1* gene does not seem to be a target of *P. indica* in this species (Figs. 4 and 5). Consistent with previous observations (Maher and Martindale 1980), we observed that loss-of-function *AUX1* and *AUX1/AXR4* seedlings are smaller than wild-type seedlings (data not shown). However in the presence of the fungus, the loss-of-function mutants showed a 45 ± 6% (*AUX1*) and 44 ± 5% (*AUX1/AXR4*) (*n* for all assays = 10 independent experiments with 20 seedlings each) increase in fresh weights, which is comparable with the wild type (41 ± 8%; *n* = 10). This indicates that AUX1-independent auxin transport is sufficient to trigger *P. indica*-induced growth promotion in *Arabidopsis*. As expected from the results in Figures 1D and 2, also *RHD6* seedlings which are defected in root hair initiation responded

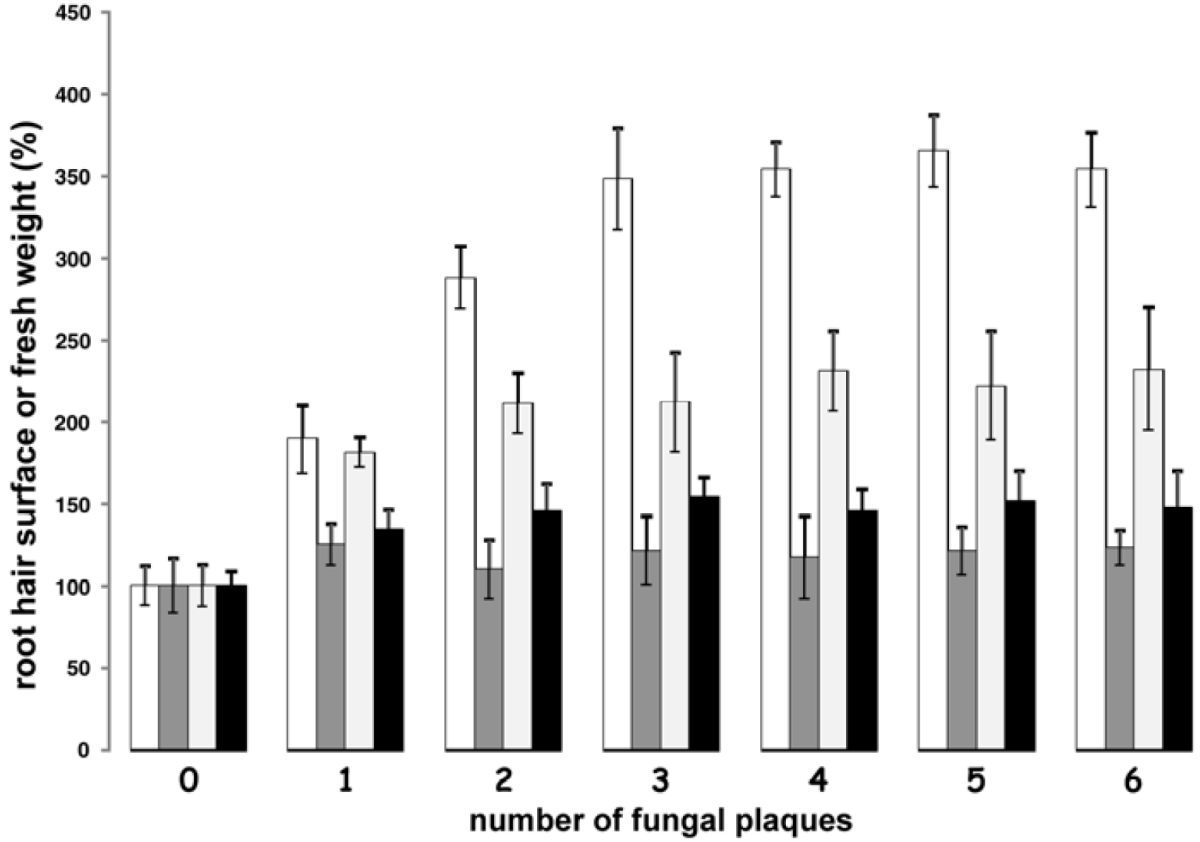


Fig. 2. Root hair surface and fresh weight of Chinese cabbage and *Arabidopsis* seedlings which were co-cultivated with 0 to 6 fungal plaques/seedling for 7 days. One seedling was grown in a petri dish. The 0 values (= control, without *Piriformospora indica*) was taken as 100 and the other values are expressed relative to it. Left two bars: root hair surface of Chinese cabbage and *Arabidopsis* seedlings, respectively, as measured by pixel numbers on an electronic photograph. Right two bars: fresh weights of Chinese cabbage and *Arabidopsis* roots, respectively. Bars represent standard errors, based on six independent experiments.

to the fungus (41 ± 7%; n = 10). Thus, *P. indica*-induced growth promotion of *Arabidopsis* seedlings is independent of AUX1 and RHD6.

A fungal exudate fraction, but not auxin, promotes growth of *Arabidopsis* and Chinese cabbage seedlings.
As reported previously, a cell wall extract from the fungus stimulates growth of *Arabidopsis* seedlings (Vadassery et al. 2009). We observe the same for Chinese cabbage seedlings (Fig. 6). The cell wall extract also stimulated growth of *AUX1, AUX1/AXR4*, and *RHD6* seedlings, while two previously identified *P. indica-insensitive* (*pii*) mutants, called *pii-3* and *pii-4*, did not respond (Vadassery et al. 2009; data not shown). The active compounds in the cell wall extract were further enriched by high-performance liquid chromatography (HPLC). The resulting fraction had the same growth-promoting features as the cell wall extract and contained no auxin (below detection limits, at least 10^5-fold less auxin than in the cell wall extract before HPLC fractionation). Growth promotion of Chinese cabbage and *Arabidopsis* seedlings could not be achieved by auxin exogenously applied to the roots (data not shown). These results demonstrate that *P. indica*-induced growth promotion in Chinese cabbage and *Arabidopsis* seedlings is not caused by fungus-released auxin.

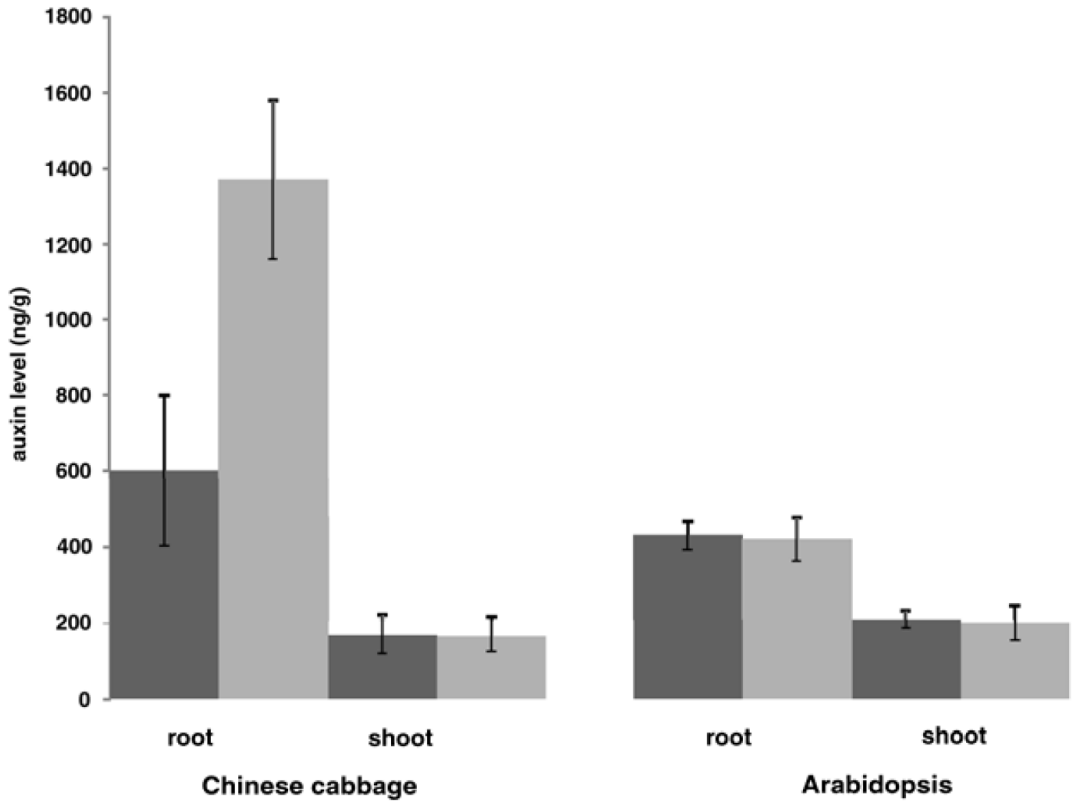


Fig. 3. Auxin levels in root and leaf tissues of Chinese cabbage and *Arabidopsis* seedlings, grown in the presence (light bars) or absence (dark bars) of *Piriformospora indica*. Leaf and root tissues were separately harvested from plants at 7 days after infection by *P. indica*.

Table 1. Annotated expressed sequence tag sequences related to auxin function

Class, gene annotation[a]	Reverence organism	GI number	*E* value	Frequency
Class 1				
AUX1 (AUXIN RESISTANT 1); amino acid transmembrane transporter/transport	*Arabidopsis thaliana*	18404642	1.00E-47	1
Efflux carrier, pin3	*Brassica juncea*	15485155	2.00E-96	1
Auxin transport protein; BIG	*A. thaliana*	21779966	2.00E-41	1
Class 2				
Vacuolar-type H^+-ATPase subunit B2	*A. thaliana*	62321641	9.00E-66	1
Pyrophosphate-energized vacuolar membrane proton pump	*Thellungiella salsuginea*	60476796	3.00E-111	2
Class 3				
Indole-acetic acid 7 (IAA7)–auxin-responsive AUX and IAA family member	*Zea mays*	195635917	1.00E-38	1
F-box and leucine-rich repeat protein 15	*A. thaliana*	124007179	9.00E-110	1
E2 ubiquitin-conjugating enzyme UBC10	*B. napus*	183013548	2.00E-59	1
Ubiquitinating enzyme	*A. thaliana*	66354424	9.00E-71	1
26S proteasome subunit 4-like protein	*B. napus*	11045086	2.00E-102	1
26S proteasome AAA-ATPase subunit RPT4a	*A. thaliana*	6652884	3.00E-62	1
26S proteasome regulatory complex subunit p42D, putative	*A. thaliana*	15219503	9.00E-61	1
Auxin-regulated protein-like protein	*Populus trichocarpa*	109676318	6.00E-05	1

[a] Class 1: auxin transport carrier; class 2: cell wall acidification; class 3: auxin signal transduction.

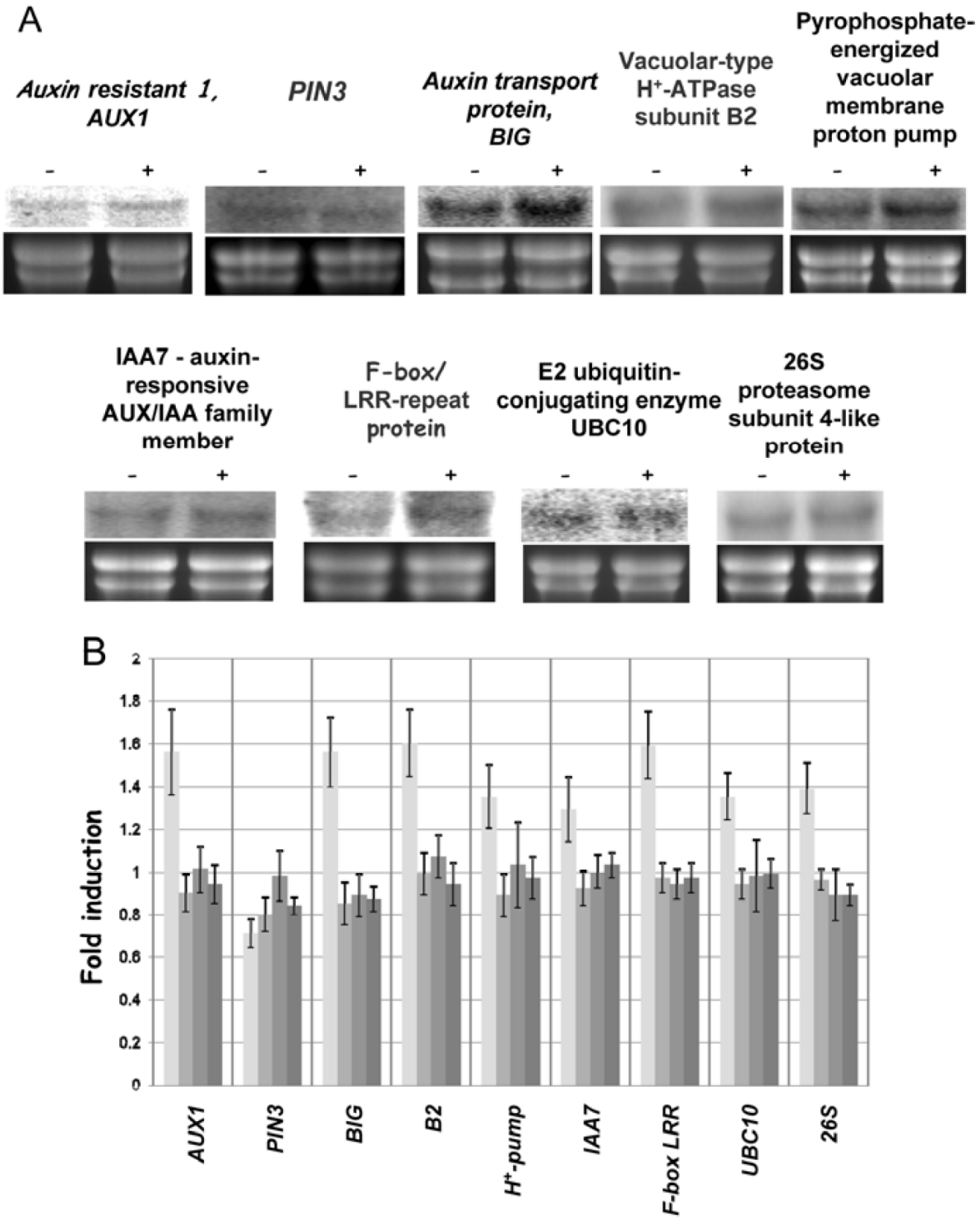


Fig. 4. A, Northern blot analysis for auxin-related genes in Chinese cabbage co-cultivated wit *Piriformospora indica* compared with untreated controls. Root tissues 7 days after infection by *P. indica* were sampled for total RNA extraction. The RNA was resolved on a formaldehyde-denatured agarose gel, and hybridized to the indicated cDNA probes. **B,** Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses for auxin-related genes from Chinese cabbage and *Arabidopsis* roots, co-cultivated with *P. indica* in the same petri dish. Lightest bar: qRT-PCR analysis for the same genes in Chinese cabbage roots, as shown in A. Based on three independent experiments. Bars represent standard errors (SE). Second lightest bar: qRT-PCR for the *Arabidopsis* homologs of the auxin-related Chinese cabbage genes in *Arabidopsis* roots. Based on three independent experiments. Bars represent SE. Dark gray bars: qRT-PCR of the auxin-related genes in Chinese cabbage roots after the application of a cell wall extract (lighter bars) or a water-diffusible fraction (darker bars) from *P. indica* to the roots. Based on three independent experiments. Bars represent SE.

DISCUSSION

Auxin plays a crucial role in Chinese cabbage–*P. indica* interaction.

Auxins play multiple roles in plant–microbe interactions (Spaepen et al. 2007); for example, by the formation of new patterns and organs (Devos et al. 2005; Grunewald et al. 2009; Tanaka et al. 2006), growth (Contreras-Cornejo et al. 2009), or defense responses (Bari and Jones 2009; Kazan and Manners 2009). Plant-growth-promoting microbes, including *P. indica*, produce auxins, which may be active in plants (Contreras-Cornejo et al. 2009; Devos et al. 2005; Sirrenberg et al. 2007;

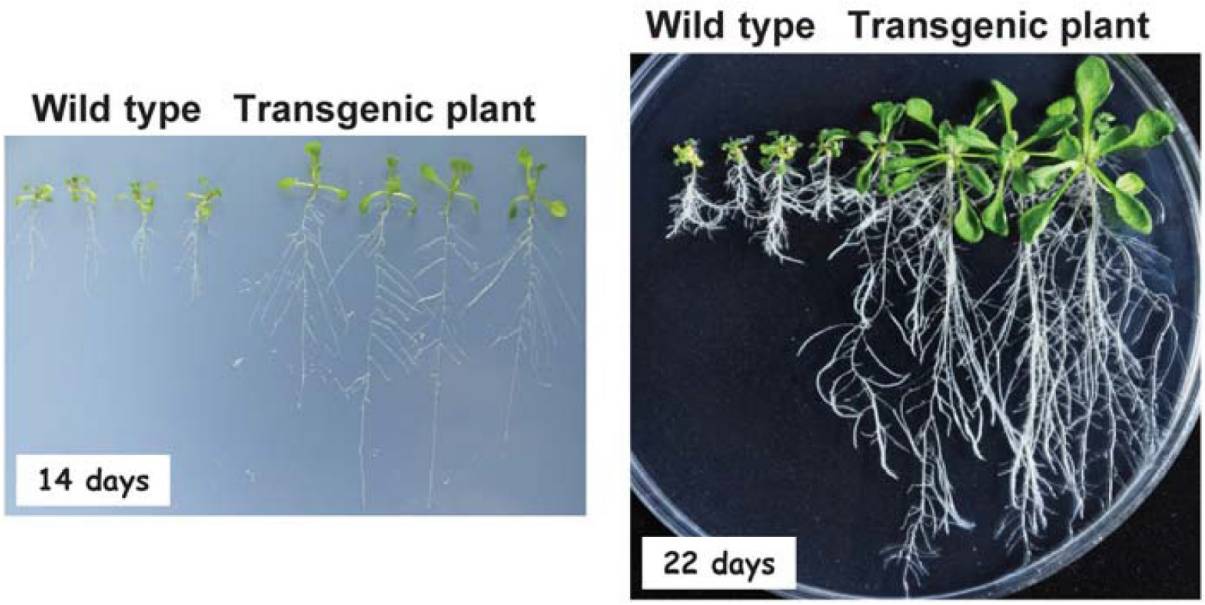


Fig. 5. Root architecture and biomass of T2 transgenic *Arabidopsis* plants overexpressing *BcAUX1*. Shown are 14- and 22-day old seedlings of wild-type (left) and transgenic *Arabidopsis* after germination on $^{1}/_{2}$ Murashige-Skoog medium.

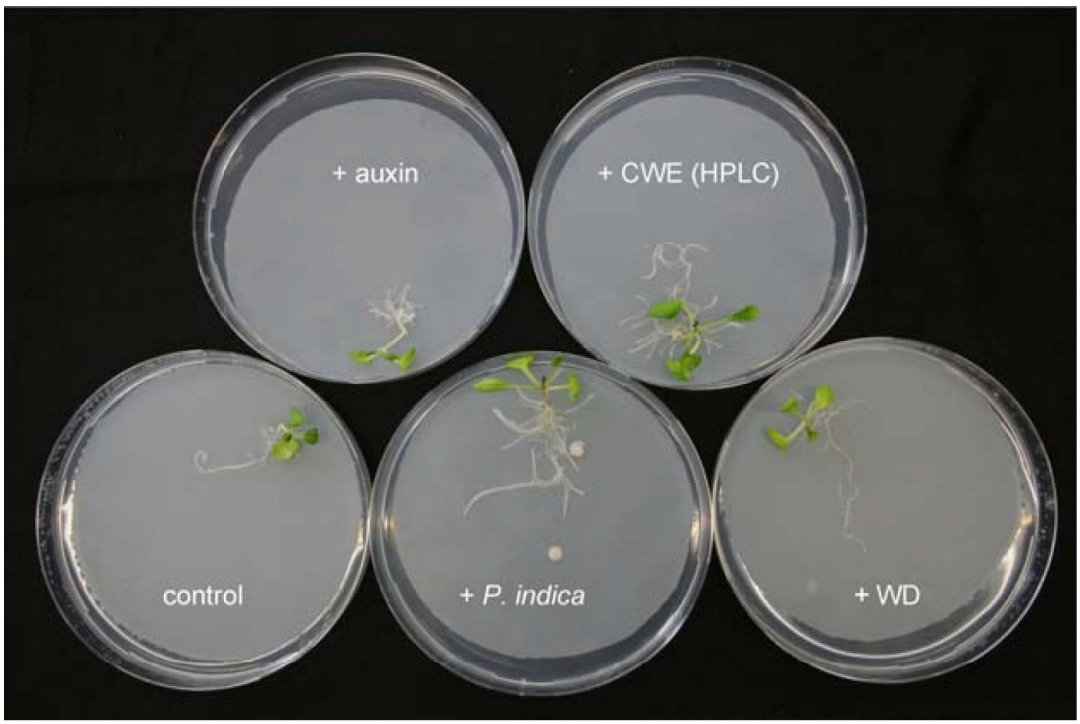


Fig. 6. Chinese cabbage seedlings which were treated with auxin (3 ppm), the high-performance liquid chromatography-purified cell wall extract (HPLC-CWE, 100 µl), a water-diffusible (WD, 100 µl) exudate fraction, or mock-treated (water, control) for 7 days on petri dishes; + *Piriformospora indica* = treatment with a fungal plaque.

Splivallo et al. 2009). Microbes also interfere with the plant auxin synthesis, metabolism, signaling, and transport (Grunewald et al. 2009; Vadassery et al. 2008) or they affect the phytohormone balance (Splivallo et al. 2009). Infection of Chinese cabbage by *Plasmodiophora brassicae* leads to a stimulation of plant growth: The auxin increase, together with an increased xyloglucan endotransglucosylase/hydrolase action, results in wall loosening and, consequently, cell expansion (Devos et al. 2005). The different distribution of the hormone within plant tissues and the establishment of gradients are important for developmental processes induced by auxins or root-colonizing microbes (Sorefan et al. 2009). Here, we demonstrate that *Piriformospora indica*-induced growth promotion in Chinese cabbage is associated with severe alterations in the root morphology (Fig. 1; Table 1) such as the promotion of adventitious root formation and root branching, processes which are controlled by auxins (Druege et al. 2007; Sirrenberg et al. 2007). Therefore, it is not surprising that co-cultivation of the two symbionts results in an increased auxin level and the upregualtion of auxin-related genes in the roots (Fig. 4).

The distribution of auxin is crucial for lateral root initiation and root development in *Arabidopsis* (Lucas et al. 2008). The polarized transport of auxin into and out of cells allows the control of cellular auxin levels and the generation of auxin gradients. Cellular auxin levels are controlled by influx and efflux carriers. AUX1 efficiently regulates auxin uptake and the expression of *AUX1* is upregulated in colonized Chinese cabbage roots (Fig. 4). PIN3, one of the efflux carriers of auxin, which is downregulated at the mRNA level in this symbiosis (Fig. 4), acts as a negative regulator of root hair growth, and overexpression of *pin3* in tobacco represses root hair development (Lee and Cho 2006). Therefore, regulation of *AUX1* and *PIN3* by *P. indica* should result in a higher auxin level in the root cell, which leads to the activation of the $SCF^{TIR1/AFBs}$ cascade genes. Consequently, a variety of physiological responses are induced, such as root growth, branching, and root hair emergence. The highly branched root phenotype of the *Arabidopsis BcAUX1* overexpressor (Fig. 5) also suggests that the morphology in colonized Chinese cabbage roots is caused by the higher *AUX1* mRNA level. Elevation of the cellular hormone level stabilizes the interaction between TIR1/AFBs and AUX/IAA proteins. In association with $SCF^{TIR1/AFBs}$, AUX/IAA become ubiquinated by the 26S proteasome for proteolysis, which results in ARF derepression and modulation of transcription of auxin-regulated genes. Many loss-of-function mutations in the components of SCF assembly in *Arabidopsis* are impaired in various auxin responses (Mockaitis and Estelle 2008). Furthermore, the expression of genes for the vacuolar-type H^+-ATPase subunit B2 and the pyrophosphate-energized vacuolar membrane proton pump was induced in infected Chinese cabbage plants (Fig. 4A and B). It has been shown for maize seedlings that even low concentrations of exogenously applied IAA (10^{-10} and 10^{-15} M) present in the soil of a field could activate a vacuolar H^+-ATPase and an H^+-pyrophosphatase, which promotes lateral root formation (Zandonadi et al. 2007). The concerted activation of the plasmalemma and tonoplast H^+-pumps by auxins and other environmental stimuli plays a key role in root cell expansion by generating an H^+ electrochemical gradient which maintains the osmotic pressure of the vacuole sufficiently high enough for water uptake and vacuolation (Maeshima et al. 1996; Smart et al. 1998). According to the acid growth theory, maintenance of the vacuolar turgor is the driving force for volume expansion and, thus, cell elongation (Cosgrove 2000). Therefore, the upregulation of genes involved in proton pumping in the infected Chinese cabbage is not surprising.

Although the auxin-related genes isolated from the double-subtracted EST library are not complete to cover all aspects, they clearly define a central role of auxin in the *P. indica*–Chinese cabbage interaction. Here, we focus on those genes which are related to auxin functions. EST related to other hormone functions such as cytokinin signaling could not be identified. Because *P. indica* produces auxin and releases small amounts into the medium, it is tempting to speculate that this auxin is responsible for these morphological changes and growth promotion. However, several observations prompted us to investigate this in greater detail.

Vadassery and associates (2008) performed similar co-cultivation experiments with *Arabidopsis* and *P. indica* and came to the conclusion that neither the auxin levels nor auxin-related genes were significantly upregulated in colonized roots. Why is the response of the two species toward *P. indica* different? The aerial parts of Chinese cabbage cultivars are optimized to produce large amounts of biomass within a short period of time, which can best be facilitated by optimal water and nutrient uptake from the soil. Therefore, *P. indica* may increase the active auxin level to enlarge the root surface and promote root hair development, in particular in response to growth-promoting stimuli. Compared with Chinese cabbage, growth stimulation is less important and not a primary developmental strategy of *Arabidopsis*. What is the origin of the additional auxin in the Chinese cabbage roots? Because exogenously applied auxin does not stimulate growth of Chinese cabbage seedlings, whereas a fungal exudate which does not contain auxin induces growth, auxin synthesized by the fungus is probably not responsible for the plant response. A comparable situation has been described for Chinese cabbage (*B. rapa* L.) roots infected by the clubroot disease-inducing *Plasmodiophora brassicae*. Upon infection by the obligate biotroph, the auxin level in the roots increases. This auxin is presumably synthesized by the plant nitrilase. Ando and associates (2008) have shown that transcriptional regulation of one gene for the nitrilase from *B. rapa*, *BrNIT2*, is involved in auxin overproduction during clubroot development.

Fungal exudates trigger plant growth.

Because an exudate fraction from *Piriformospora indica* without detectable auxin promotes growth of *Arabidopsis* and Chinese cabbage seedlings, a living fungus is not required for growth promotion. The HPLC-purified exudate fraction contains no sucrose or other sugars; therefore, those metabolites as growth-promoting substances can also be excluded. It remains to be determined which components in the fungal exudates are responsible for the plant response and what are their targets in the root cell. We propose that *P. indica*-associated molecular patterns promote growth of Chinese cabbage by interfering with the auxin homeostasis. Higher auxin levels in colonized Chinese cabbage roots indicate that the fungal signals induce auxin biosynthesis or its release from conjugates. Regulation of auxin transporter genes also suggests that auxin homeostasis may also be influenced through interference with its transport.

AUX1 is a growth regulator.

We identified *AUX1* as a target gene of *P. indica* in Chinese cabbage roots. Overexpression of *BcAUX1* in *Arabidopsis* demonstrates that this protein is an important growth regulator in *Arabidopsis*, although it is not targeted by the fungus in this species. Growth promotion by *P. indica* or fungal exudates in *Arabidopsis* seedlings is not dependent on AUX1, because *aux1* and *aux1/axr4* deletion lines respond to the fungus. Similarly, Splivallo and associates (2009) have shown that truffles promote root growth of *aux1-7* seedlings by fungus-derived metabolites. Furthermore, *rhd6* seedlings which are impaired in root hair initiation also responded to the fungus, consistent with the results that root hair development and, apparently, nu-

trient uptake from agar plates via the root hairs is not limiting for the response to *P. indica*.

The AUX1 protein sequences of both species differ only in their extreme N and C termini while the middle part is almost completely conserved. Therefore, it is likely that the growth-promoting function of AUX1 is not restricted to the Chinese cabbage protein. Because the stimulatory effect cannot be achieved by exogenous application of auxin, AUX1 appears to fulfill rate-limiting functions in auxin transport or signaling in both species. The larger root system, including the increase in lateral roots in the overexpressor line, may provide advantages for the plants when grown under nutrient limitations, and it could affect plant performance, depending on the microbial communities in the rhizosphere. How the growth response of the aerial parts is related to the manipulation of AUX1 remains to be investigated. Finally, comparison of *P. indica*- and AUX1-mediated effects on plant performance may help to identify target genes useful for biotechnological applications.

MATERIALS AND METHODS

Growth conditions of plants and fungus, co-cultivation experiments, and estimation of plant growth.

Seed of Chinese cabbage (*B. campestris* subsp. *chinensis*) were donated from the Ming-Hong Seed Company, Feng-Yuan City, Taiwan. Seeds were surface-sterilized with 75% alcohol for 10 min, then placed on a petri dish containing $^1/_2$ MS nutrient medium (Murashige and Skoog 1962). Plates were incubated at 22°C under continuous illumination (100 µmol m^{-2} s^{-1}) for seed germination. *Arabidopsis* (ecotype Columbia) seedlings were germinated as described by Vadassery and associates (2009) and grown on petri dishes containing $^1/_2$ MS nutrient medium, identical to the Chinese cabbage seedlings. The homozygote *aux1* (N9583), *aux1/axr4* (N8040), and *rhd6* (N6347) lines were obtained from NASC (Hobbie and Estelle 1995).

Seven days after seed plating on $^1/_2$ MS medium, the growing seedlings were transferred to fresh plates containing $^1/_2$ MS medium. One to six seedlings were used per petri dish and one fungal plague or one agar plague without fungus of 5 mm in diameter per seedling was placed at a distance of 1 cm from the roots. If more than one seedling was used in the co-cultivation experiment in one petri dish, this is mentioned in the figure legend or visible in the figure. For the results shown in Figure 2, the number of fungal or agar plaques or seedlings was varied. For direct comparison of Chinese cabbage and *Arabidopsis* seedlings, both seedlings were grown in the same petri dish, with a fungal or agar plaque positioned 1 cm away from each seedling. However, we did not observe any difference to seedlings which were grown on separate plates. For experiments with fungal-derived extracts, 100 µl of the extracts was applied once to the roots instead of the fungal plaques at the same position and time. IAA from 1.5 to 15 ppm served as positive control and was applied in the same way as the fungal exudates. The plates were incubated at 22°C under continuous illumination from the side (80 µmol m^{-2} s^{-1}). *P. indica* was cultured as described previously (Peškan-Berghöfer et al. 2004; Verma et al. 1998) on Kaefer medium (Hill and Kaefer 2001). Seedlings were removed from the plates at 7 days after co-cultivation for auxin measurements, analysis of the morphology, determination of the fresh weight, and RNA extraction. Alternatively, the seedlings were transferred to pots and grown in a walk-in growth chamber, as described previously (Vadassery et al. 2009).

Measurement of auxin concentration.

The auxin level of the plant tissues was analyzed by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) as described previously (Chen et al. 2008). The tissues were sampled 7 days after co-cultivation with *P. indica*. The tissue was ground in liquid N_2 with a mortar and pestle. Internal standards of 100 ng of [$^{13}C_6$]-IAA were added to each sample after grinding. Tissue extraction was carried out overnight with 15 ml of 80% (vol/vol) methanol containing 0.4 mg of butylated hydroxytoluene and 2 mg of ascorbate at 5°C. The methanol extracts were combined and reduced to 1 to 2 ml with a rotary vacuum evaporator and SpeedVac (Savant Instruments, Hicksville, NY, U.S.A.). The concentrate was adjusted to pH 8.5 with 0.05 M potassium phosphate buffer and passed through a polyvinyl polypyrrolidone column (5 g). The eluate was partitioned with ethyl acetate (3 × 15 ml). The aqueous fraction was then adjusted to pH 3.0 with 0.5 M potassium phosphate (pH 2.0) and partitioned with ethyl acetate (3 × 15 ml). The pooled ethyl acetate fraction was completely dried with a SpeedVac. The pellet was dissolved in 0.05 M potassium phosphate buffer (3 g). The ODS-silica column was washed three times with double-distilled H_2O in 0.1% acetic acid and eluted with 80% aqueous methanol containing 0.1% acetic acid.

After drying under vacuum, the sample was dissolved in 30% aqueous methanol containing 0.1% acetic acid and injected into a Beckman System Gold HPLC with a LiChrosphere RP-18 column (250 by 4 mm in diameter by 5-µm particle size (Merck, Darmstadt, Germany). The fraction of IAA from this column was dried under vacuum and derivatized by adding ethereal diazomethane, then dried with N_2. IAA was further trimethylsilated and the derivatized samples were analyzed using Agilent Technologies 6890N GC and 5973 MSD with a DB-1 capillary column (30 by 0.25 mm in diameter, 0.25-µm film thickness (J&W Scientific, Folsom, CA, U.S.A.).

Construction of subtractive EST library and analysis of EST clones.

A previously published method for pine tree (Chang et al. 1993) was used to extract total RNA from Chinese cabbage and fungal mycelium. Roots of Chinese cabbage, which was co-cultivated with *P. indica* for 7 days, were freshly sampled. Mycelium of *P. indica* cultured on Kaefer medium (Hill and Kaefer,2001) for 3 weeks was employed for total RNA extraction. mRNA was purified from total RNA with an Oligotex mRNA Kit (Qiagen, Chatsworth, CA, U.S.A.) and cDNA was synthesized by using the PCR-selected TMcDNA subtraction kit following the manufacturer's instruction (Clontech, Mountain View, CA, U.S.A.). cDNA was digested by *Rsa*I and then ligated with PCR adaptors. The double-subtracted hybridization was performed by using cDNAs of infected plant roots as tester. cDNAs of uninfected plant roots and of fungal mycelium were used together as drivers. According to the manufacturer's instructions, the subtracted cDNA mixture was amplified by PCR once and the products were cloned into the pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.) with blue and white selection in *Escherichia coli* XL1-Blue. Three hundred white clones were randomly selected and cultured in Luria-Bertani medium at 37°C overnight. Plasmid DNA was extracted and the insertions were sequenced. The EST sequences were assembled to obtain contigs and singletons. To annotate the clusters and singles, sequence alignment was performed by BlastX (Altschul et al. 1977) with the nonredundant protein sequence database in GenBank (National Center for Biotechnology Information) with an *E* value threshold of E-10.

Analysis of gene expression in Chinese cabbage roots by Northern blot hybridization.

Total RNA (10 µg each) from infected root tissues and control tissue was loaded on 1% agarose/formaldehyde gels and transferred onto nylon membranes (Amersham Bioscience, Piscataway, NJ, U.S.A.). The EST insert fragments, randomly

labeled with α-^{32}P-dCTP (Rediprime II Kit; Amersham Bioscience), were employed as probe to detect the expression pattern. The membrane hybridization and fluorescent signal detection (Typhoon 9400; Amersham Bioscience) were carried out following standard molecular protocols. The *ACTIN2* gene from *Arabidopsis* was used as loading control (data not shown).

Analysis of gene expression by qRT-PCR.

RNA was isolated from roots of Chinese cabbage and *Arabidopsis* seedlings with an RNA isolation kit (RNeasy, Qiagen, Hilden, Germany) and reverse-transcribed for real-time qPCR analysis using an iCycler iQ real-time PCR detection system and iCycler software (version 2.2; Bio-Rad, Munich). Total RNA was isolated from three independent replicates of the roots. cDNA was synthesized using the Omniscript cDNA synthesis kit (Qiagen, Hilden, Germany) using 1 μg of RNA. For the amplification of the RT-PCR products, iQ SYBR Green Supermix (Bio-Rad, Hilden, Germany) was used according to the manufacturer's protocol in a final volume of 25 μl. The iCycler was programmed to 95°C for 2 min; 40 cycles of 95°C for 30 s, 57°C for 40 s, and 72°C for 45 s; 72°C for 10 min; followed by a melting curve program of 55 to 95°C in increasing steps of 0.5°C. All reactions were performed in triplicate. The mRNA levels for each cDNA probe were normalized with respect to the *ACTIN2* mRNA level. Fold induction values of target genes were calculated with the ΔΔCP equation of Pfaffl (2001) and related to the mRNA level of target genes in control roots, which were defined as 1.0. The primer pairs for product sizes between 150 and 170 bp are given in Supplementary Figure 1.

Detection of fungal RNA in Chinese cabbage roots has been performed as described previously (Bütehorn et al. 2000).

5′ and 3′ RACE for the generation of the full-length AUX1 gene from Chinese cabbage.

The RACE experiments were performed according to the manufacturer's instructions (InVitrogene, Karlsruhe, Germany).

***A. tumefaciens* transformation.**

Arabidopsis transformation was performed according to Bechtold and associates (1993).

Preparation of a water-diffusible fraction from *P. indica*.

P. indica mycelium, propagated in liquid culture medium for 2 weeks, was filtered through eight layers of nylon membrane, collected in a funnel, and intensively washed seven times with distilled water. After air drying of the mycelium, it was resuspended in distilled water. After 48 h, the mycelium was removed from the water by high-speed centrifugation and the supernatant was used for the experiments (=water-diffusible fraction).

Preparation of an extract from the cell-wall fraction from *P. indica*.

The cell wall extract was prepared using the protocol of Anderson-Prouty and Albersheim (1975) with modifications. Mycelia from 14-day-old liquid cultures were homogenized using a Waring blender in 5 ml of water per gram of mycelia. The homogenate was filtered through eight layers of nylon membrane and a coarse-sintered glass funnel. The residue was washed three times with water, twice with chloroform/methanol (1:1), and, finally, twice in acetone. This preparation was air dried for 2 h and the mycelial cell wall material was recovered. Elicitor fractions were prepared from mycelial cell walls by suspending 1 g of cell wall material in 100 ml of water and autoclaving for 30 min at 121°C. Autoclaving releases the active fraction. After filtration through nylon membranes, the suspension was centrifuged at 14,000 rpm for 10 min and filter-sterilized using a 0.22-μM filter. The extract was further purified by passing it through a reverse-phase Superclean LC-18 Cartridge (Sigma-Aldrich, Taufkirchen, Germany). The active fractions were collected and concentrated to half.

Statistics.

Statistics were performed by one-way analysis of variance, and the experiments were repeated four times, with values represented by standard errors.

ACKNOWLEDGMENTS

We thank the National Science Council of the Republic of China and the Deutscher AkademischerAustausch Dienstof Germany (NSC-DAAD) for their financial support for PPP project NSC-97-2911-1-002-010-2 to K.-W. Yeh and D. Cai. Thanks are also due to the National Taiwan University for partial financial support of international collaboration funding to K.-W. Yeh under project no. 98R0705.

LITERATURE CITED

Altschul, S. F., Madden, L., Schaffer, A. A., Zhang, Z., Miller, W., and Lipman, D. 1977. Gapped BLAST and SI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Anderson-Prouty, A. J., and Albersheim, P. 1975. Host–pathogen interactions. VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogens host. Plant Physiol. 56:286-291.

Ando, S., Tsushima, S., Kamachi, S., Konagaya, K., and Tabei, Y. 2008. Alternative transcription initiation of the nitrilase gene (BrNIT2) caused by infection with *Plasmodiophora brassicae* Woron. in Chinese cabbage (*Brassica rapa* L.). Plant Mol. Biol. 68:557-669.

Bari, R., and Jones, J. D. 2009. Role of plant hormones in plant defence responses. Plant Mol. Biol. 69:473-488.

Bechtold, N., Ellis, J., and Pelletier, G. 1993. In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris Life Sci. 316:1194-1199.

Boerjan, W., Cervera, M. T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., Onckelen, H. V., Montagú, M. V., and Inzé, D. 1995. Superroot, a recessive mutation in *Arabidopsis*, confers auxin overproduction. Plant Cell 7:1405-1419.

Bütehorn, B., Rhody, D., and Franken, P. 2000. Isolation and characterisation of Pitef1 encoding the translation elongation factor EF-1α of the root endophyte *Piriformospora indica*. Plant Biol. 2:687-692.

Casimiro, I., Marchant, A., Bhalerao, R. P., Swarup, R., Graham, N., Inzé, D., Sandberg, G., Casero, P. J., and Bennett, M. 2001. Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell 13:843-852.

Celenza, J. L., Grisafi, P. L., and Fink, G. R. 1995. A pathway for lateral root formation in *Arabidopsis thaliana*. Genes Dev. 9:2131-2142.

Chang, S., Puryear, J., and Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11:113-116.

Chen, S. Y., Kuo, S. R., and Chien, C. T. 2008. Roles of gibberellins and abscisic acid in dormancy and germination of red bayberry (*Myrica rubra*) seeds. Tree Physiol. 28:1431-1439.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Cortés-Penagos, C., and López-Bucio, J. 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant Physiol. 149:1579-1592.

Cosgrove, D. J. 2000. Expansive growth of plant cell walls. Plant Physiol. Biochem. 38:109-124.

Devos, S., Vissenberg, K., Verbelen, J. P., and Prinsen, E. 2005. Infection of Chinese cabbage by *Plasmodiophora brassicae* leads to a stimulation of plant growth: Impacts on cell wall metabolism and hormone balance. New Phytol. 166:241-250.

Dharmasiri, S., Swarup, R., Mockaitis, K., Dharmasiri, N., Singh, S. K., Kowalchyk, M., Marchant, A., Mills, S., Sandberg, G., Bennett, M. J., and Estelle, M. 2006. AXR4 is required for localization of the auxin influx facilitator AUX1. Science 12:1218-1220.

Druege, U., Baltruschat, H., and Franken, P. 2007. *Piriformospora indica* promotes adventitious root formation in cuttings. Sci. Hortic. 112:422-426.

Estelle, M., and Somerville, C. 1987. Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol. Gen. Genet. 206:200-206.

Feraru, E., and Friml, J. 2008. PIN polar targeting. Plant Physiol. 147:1553-1539.

Friml, J. 2003. Auxin transport—shaping the plant. Curr. Opin. Plant Biol. 6:7-12.

Fukaki, H., and Tasaka, M. 2009. Hormone interactions during lateral root formation. Plant Mol. Biol. 69:437-449.
Gil, P., Dewey, E., Friml, J., Zhao, Y., Snowden, K. C., Putrill, J., Palme, K., Estelle, M., and Chory, J. 2001. BIG: A calossin-like protein required for polar auxin transport in *Arabidopsis*. Genes Dev. 15:1985-1997.
Gray, W. M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. 2001. Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature 414:271-276.
Grunewald, W., van Noorden, G., Van Isterdael, G., Beeckman, T., Gheysen, G., and Mathesius, U. 2009. Manipulation of auxin transport in plant roots during Rhizobium symbiosis and nematode parasitism. Plant Cell 21:2553-2562.
Gupta, A., Gopal, M., and Tilak, K. V. 2000. Mechanism of plant growth promotion by rhizobacteria. Indian J. Exp. Biol. 38:856-862.
Hill, T. W., and Kaefer, E. 2001. Improved protocols for *Aspergillus* medium: Trace elements and minimum medium salt stock solutions. Fungal Genet. Newsl. 48:20-21.
Hobbie, L., and Estelle, M. 1995. The *AXR4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. Plant J. 7:211-220.
Kazan, K., and Manners, J. M. 2009. Linking development to defense: Auxin in plant–pathogen interactions. Trends Plant Sci. 14:373-382.
King, J. J., Stimart, D. P., Fisher, R. H., and Bleecker, A. B. 1995. A mutation altering auxin homeostasis and plant morphology in *Arabidopsis*. Plant Cell 7:2023-2037.
Lee, S. H., and Cho, H. T. 2006. PINOID positively regulates auxin efflux in *Arabidopsis* root hair cells and tobacco cells. Plant Cell 18:1604-1616.
Lucas, M., Godin, C., Jay-Allemand, C., and Laplaze, L. 2008. Auxin fluxes in the root apex co-regulate gravitropism and lateral root initiation. J. Exp. Bot. 59:55-66.
Ludwig-Müller, J. 1999. The biosynthesis of auxins. Curr. Top. Plant Biol. 1:77-88.
Ludwig-Müller, J. 2000. Hormonal balance in plants during colonization by mycorrhizal fungi. Pages 263-285 in: Arbuscular Mycorrhizas: Physiology and Function. D. D. Douds and Y. Kapulnik, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Ludwig-Müller, J., Vertocnik, A., and Town, C. D. 2005. Analysis of indole-3-butyricacid-induced adventitious root formation on *Arabidopsis* stem segments. J. Exp. Bot. 56:2095-2105.
Luschnig, C., Gaxiola, R. A., Grisafi, P., and Fink, G. R. 1998. EIR1, a root-specific protein involved in auxin transport is required for gravitropism in *Arabidopsis thaliana*. Genes Dev. 12:2175-2187.
Maeshima, M., Nakanishi, Y., Matsuura-Endo, C., and Tanaka, Y. 1996. Proton pumps of the vacuolar membrane in growing plant cells. J. Plant Res. 109:119-125.
Maher, E. P., and Matindale, S. J. B. 1980. Mutants of *Arabidopsis thaliana* with altered responses to auxin and gravity. Biochem. Genet. 18:1041-1053.
Masucci, J. D., and Schiefelbein, J. W. 1994. The *RHD6* mutation of *Arabidopsis thaliana* alters root hair initiation through an auxin and ethylene associated process. Plant Physiol. 106:1335-1346.
Mockaitis, K., and Estelle, M. 2008. Auxin receptor s and plant development: A new signaling paradigm. Annu. Rev. Cell. Dev. Biol. 24:55-80.
Montesinos, E., Bonaterra, A., Badosa, E., Francés, J., Alemany, J., Llorente, I., and Moragrega, C. 2002. Plant–microbe interactions and the new biotechnological methods of plant disease control. Int. Microbiol. 5:169-175.
Morgan, J. A., Bending, G. D., and White, P. J. 2005. Biological costs and benefits to plant–microbe interactions in the rhizosphere. J. Exp. Bot. 56:1729-1739.
Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15:473-497.
Oelmüller, R., Shahollari, B., Peškan-Berghöfer, T. T., Giong, P. H., Sherameti, I., Oudhoff, M., Venus, Y., Altschmied, L., and Varma, A. 2004. Molecular analysis of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformospora indica*. Endocyt. Cell Res. 15:504-517.
Oelmüller, R., Sherameti, I., Tripathi, S., and Varma, A. 2009. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1-17.
Oono, Y., Chen, Q. G., Overvoorde, P. J., Köhler, C., and Theologis, A. 1998. Age mutants of *Arabidopsis* exhibit altered auxin-regulated gene expression. Plant Cell 10:1649-1662.
Paponov, I. A., Teale, W. D., Trebar, M., Blilou, I., and Palme, K. 2005. The PIN auxin efflux facilitators: Evolutionary and functional perspectives. Trends Plant Sci. 10:170-177.
Peškan-Berghöfer, T., Shahollari, B., Giang, P. H., Hehl, S., Markert, C., Blanke, V., Varma, A. K., and Oelmüller, R. 2004. Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. Physiol. Plant. 122:465-477.
Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45.
Pieterse, C. M., and Dicke, M. 2007. Plant interactions with microbes and insects: From molecular mechanisms to ecology. Trends Plant Sci. 12:564-569.
Raven, J. A. 1975. Transport of indoleacetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. New Phytol. 74:163-172.
Reed, R. C., Brady, S. R., and Muday, G. 1998. Inhibition of auxin movement from the shoot into the root inhibits lateral root development in *Arabidopsis*. Plant Physiol. 118:1369-1378.
Sahay, N. S., and Varma, A. 1999. *Piriformospora indica*: A new biological hardening tool for micropropagated plants. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 181:297-302.
Schäfer, P., Pfiffi, S., Voll, L. M., Zajic, D., Chandler, P. M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U., and Kogel, K. H. 2009. Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. Plant J. 59:461-474.
Sirrenberg, A., Gobel, C., Grond, S., Czempinski, N., Ratzinger, A., Karlovski, P., Santos, P., Feussner, I., and Pawlowski, K. 2007. *Piriformospora indica* affects plant growth by auxin production. Physiol. Plant. 131:581-589.
Smart, L. B., Vojdani, F., Maeshima, M., and Wilkins, T. A. 1998. Genes involved in osmoregulation during turgor-driven cell expansion of developing cotton fibres are differentially regulated. Plant Physiol. 116:1539-1549.
Sorefan, K., Girin, T., Lijegren, S. J., Ljung, K., Robles, P., Galvan-Ampudia, C. S., Offringa, R., Friml, J., Yanofsky, M. F., and Ostergaard, L. 2009. A regulated auxin minimum is required for seed dispersal in *Arabidopsis*. Nature 459:583-586.
Spaepen, S., Vanderleyden, J., and Remans, R. 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 3:425-448.
Splivallo, R., Fischer, U., Göbel, C., Feussner, I., and Karlovsky, P. 2009. Truffles regulate plant root morphogenesis via the production of auxin and ethylene. Plant Physiol. 150:2019-2028.
Sun, C., Johnson, J. M., Cai, D., Sherameti, I., Oelmüller, R., and Lou. B. 2010. *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. J. Plant Physiol. 167:1009-1017.
Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K., and Bennett, M. 2001. Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. Genes Dev. 15:2648-2653.
Tanaka, H., Dhonukshe, P., Brewer, P. B., and Friml, J. 2006. Spatiotemporal asymmetric auxin distribution: A means to coordinate plant development. Cell. Mol. Life Sci. 63.:2738-2754.
Ulmasov, T., Liu, Z. B., Hagen, G., and Guilfoyle, T. J. 1995. Composite structure of auxin response elements. Plant Cell 7:1611-1623.
Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T. 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9:1963-1971.
Vadassery, J., Ritter, C., Venus, Y., Camehl, I., Varma, A., Shahollari, B., Novák, O., Strnad, M., Ludwig-Müller, J., and Oelmüller, R. 2008. *Piriformospora indica*-mediated growth promotion in *Arabidopsis* is sensitive to high auxin levels, requires trans-cytokinin biosynthesis and the cytokinin receptor combination CRE1/AHK2. Mol. Plant-Microbe Interact. 21:1371-1383.
Vadassery, J., Ranf, S., Drzewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J., and Oelmüller, R. 2009. A cell wall extract from *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J. 59:193-206.
Vanneste, S., and Friml, J. 2009. Auxin: A trigger for change in plant development. Cell 136:1005-1016.
Verma, S. A., Varma, A., Rexer, K.-H., Hassel, A., Kost, G., Sarbhoy, A., Bisen, P., Bütehorn, B., and Franken, P. 1998. *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:898-905.
Zandonadi, D. B., Canellas, L. P., and Façanha, A. R. 2007. Indoleacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H+ pumps activation. Planta 225:1583-1595.

4.2 Manuscript II

Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*

Pyniarlang L. Nongbri*, Joy Michal Johnson*, Irena Sherameti, Erich Glawischnig, Barbara Ann Halkier and Ralf Oelmüller

Molecular Plant Microbe Interaction (2012), 25(9): 1186-1197.
(*) contributed equally

MPMI Vol. 25, No. 9, 2012, pp. 1186–1197. http://dx.doi.org/10.1094/MPMI-03-12-0071-R. © 2012 The American Phytopathological Society e-Xtra*

Indole-3-Acetaldoxime-Derived Compounds Restrict Root Colonization in the Beneficial Interaction Between *Arabidopsis* Roots and the Endophyte *Piriformospora indica*

Pyniarlang L. Nongbri,[1] Joy Michal Johnson,[1] Irena Sherameti,[1] Erich Glawischnig,[2] Barbara Ann Halkier,[3] and Ralf Oelmüller[1]

[1]Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, 07743 Jena, Germany; [2]Lehrstuhl für Genetik, Technische Universität München, Emil-Ramann-Str.8, 85350 Freising, Germany; [3]VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

Submitted 29 March 2012. Accepted 17 May 2012.

The growth-promoting and root-colonizing endophyte *Piriformospora indica* induces camalexin and the expression of *CYP79B2*, *CYP79B3*, *CYP71A13*, *PAD3*, and *WRKY33* required for the synthesis of indole-3-acetaldoxime (IAOx)-derived compounds in the roots of *Arabidopsis* seedlings. Upregulation of the mRNA levels by *P. indica* requires cytoplasmic calcium elevation and mitogen-activated protein kinase 3 but not root-hair-deficient 2, radical oxygen production, or the 3-phosphoinositide-dependent kinase 1/oxidative signal-inducible 1 pathway. Because *P. indica*–mediated growth promotion is impaired in *cyp79B2 cyp79B3* seedlings, while *pad3* seedlings—which do not accumulate camalexin—still respond to the fungus, IAOx-derived compounds other than camalexin (e.g., indole glucosinolates) are required during early phases of the beneficial interaction. The roots of *cyp79B2 cyp79B3* seedlings are more colonized than wild-type roots, and upregulation of the defense genes *pathogenesis-related* (*PR*)*-1*, *PR-3*, *PDF1.2*, *phenylalanine ammonia lyase*, and *germin* indicates that the mutant responds to the lack of IAOx-derived compounds by activating other defense processes. After 6 weeks on soil, defense genes are no longer upregulated in wild-type, *cyp79B2 cyp79B3*, and *pad3* roots. This results in uncontrolled fungal growth in the mutant roots and reduced performance of the mutants. We propose that a long-term harmony between the two symbionts requires restriction of root colonization by IAOx-derived compounds.

Important secondary metabolites in members of order Brassicales are synthesized from tryptophan. Two functionally redundant cytochrome P450 enzymes (CYP79B2 and CYP79B3) convert tryptophan into indole-3-acetaldoxime (IAOx), an intermediate for the biosynthesis of indole glucosinolates (I-GLS), camalexin, other indole compounds such as indole acetonitrile, indole carboxylic acid derivatives, and, under specific conditions, the plant hormone indole-3-acetic acid (IAA). Whereas camalexin is a true phytoalexin synthesized in the plant in response to pathogen infection or exposure to stress, I-GLS are both phytoanticipins present prior to induction as well as phytoalexins induced upon infection. The first committed step in I-GLS biosynthesis is the enzyme CYP83B1/SUR2 (Bak et al. 2001; Barlier et al. 2000; Hansen et al. 2001). The major IAOx-metabolizing enzyme in camalexin biosynthesis is CYP71A13, which catalyzes the formation of the intermediate indole acetonitrile (Nafisi et al. 2007), supposedly together with the homolog CYP71A12 required for camalexin exudation from roots (Millet et al. 2010). The thiazole ring of camalexin derives from the cysteine moiety of glutathione, which is conjugated with indole acetonitrile after activation (Böttcher et al. 2009; Geu-Flores et al. 2011; Parisy et al. 2007; Su et al. 2011). The single-copy gene *PAD3/CYP71B15* encodes for an enzyme that catalyzes the final two steps in the camalexin pathway (Böttcher et al. 2009; Schuhegger et al. 2006).

The double *cyp79B2 cyp79B3* mutant lacks I-GLS (Zhao et al. 2002) and is unable to induce camalexin synthesis (Glawischnig et al. 2004). Furthermore, it does not accumulate indole-3-carboxylic acid derivatives (Böttcher et al. 2009), secondary metabolites which are strongly induced by pathogen infections. IAA levels were unchanged in seedlings of this mutant (Sugawara et al. 2009), although some reduction was observed under heat stress (Zhao et al. 2002) and in root tips (Ljung et al. 2005). In summary, IAOx has an important role as a metabolic branch point regulating flux into I-GLS, camalexin, other secondary indole compounds, and IAA biosynthesis (Burow et al. 2010; Mikkelsen et al. 2009), and diversion of IAOx into one of the pathways may occur at the expense of the others (Bak et al. 2001; Glawischnig et al. 2004; Nafisi et al. 2006).

Production of camalexin is induced in response to a variety of exposures such as plant pathogens, including bacteria, fungi, and oomycetes (Glawischnig 2007; Rauhut and Glawischnig 2009), pathogen-associated molecular patterns (PAMPs), toxins (Gust et al. 2007; Qutob et al. 2006; Rauhut et al. 2009; Stone et al. 2000), and reactive oxygen species (ROS)-inducing abiotic stress (Van Breusegem et al. 2008). Camalexin exhibits cytotoxicity, particularly against eukaryotic pathogens (Rogers et al. 1996). Expression of the last enzyme of the camalexin biosynthetic pathway, PAD3, is regulated by a variety of signaling

P. L. Nongbri and J. M. Johnson contributed equally to the work.

Corresponding author: R. Oelmüller; E-mail: b7oera@uni-jena.de

*The *e*-Xtra logo stands for "electronic extra" and indicates that four supplementary tables and one supplementary figure are published online and that Figure 5 appears in color online.

pathways. Crucial components are the mitogen-activated protein kinases (MPK) MPK3, MPK6 (Ren et al. 2008), and MPK4 (Qiu et al. 2008). MPK4 becomes activated in response to infection and phosphorylates MPK4 substrate 1 (MKS1) and the transcription factor WRKY33 in the nucleus, which ultimately allows WRKY33 to activate camalexin biosynthetic genes (Qiu et al. 2008). Upstream of MPK, the 3-phosphoinositide-dependent protein kinase 1 (PDK1)/oxidative signal-inducible 1 (OXI1) pathway is activated in response to various pathogens (Anthony et al. 2004, 2006; Rentel et al. 2004). OXI1 is required for full activation of MPK3 and MPK6 in response to many microbial pathogens or elicitors (Rentel et al. 2004; van der Luit et al. 2000; Yamaguchi et al. 2005). OXI1 phosphorylates and, thus, activates the downstream serine/threonine kinase PTI1-2 and also controls root hair growth (Rentel et al. 2004). OXI1 is activated by H_2O_2 and phospholipid signals via PDK1 (Anthony et al. 2006). PDK1 binds to different signaling lipids, including the second messenger phosphatidic acid, which accumulates in response to microbial infections or elicitor treatments (Deak et al. 1999; Hirt et al. 2011). Phosphatidic acid is mainly produced by phospholipase α1 in *Arabidopsis* roots, and a corresponding mutant contains severely reduced levels of this plant second messenger (Devaiah et al. 2006). PDK1 phosphorylates and, thus, activates OXI1 in *Arabidopsis* (Anthony et al. 2004) and rice (Matsui et al. 2010) or AvrPto-dependent Pto-interacting protein 3 (Adi3) in tomato (Devarenne et al. 2006).

Root-hair-deficient 2 (RHD2) is crucial for ROS production in roots, and inactivation of *RHD2* compromises the expansion of root hair cells. Furthermore, Ca^{2+} influx is required for the cell elongation in roots, and *rhd2* mutants are defective in Ca^{2+} uptake. As a consequence, *rhd2* mutants have short root hairs and stunted roots (Foreman et al. 2003). RHD6 controls root hair initiation and the *RHD6* mutation can be rescued by auxin (Masucci and Schiefelbein 1994) or microbes producing auxin-related compounds (Contreras-Cornejo et al. 2009). The observation that ROS induction and MPK3, MPK4, and MPK6 signaling activates camalexin biosynthesis suggests that ROS-producing enzymes and the PDK1/OXI1 pathway may be upstream in the signaling pathway to induce genes for IAOx-derived compounds.

Piriformospora indica colonizes the roots of many plant species, thereby promoting growth and biomass production and conferring resistance against biotic and abiotic stress (Oelmüller et al. 2009; Peškan-Berghöfer et al. 2004). The beneficial inter-

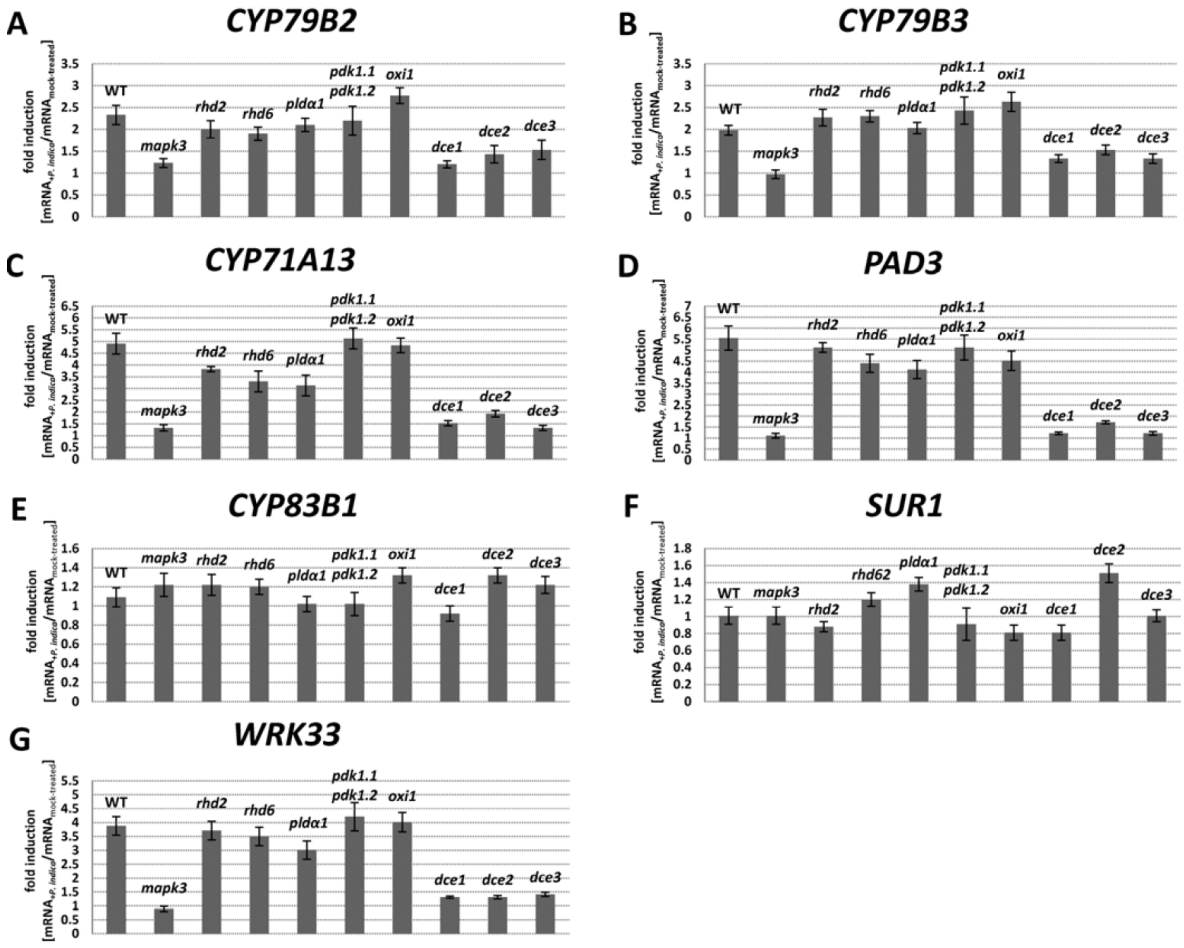


Fig. 1. Fold-induction of the mRNA levels for **A,** *CYP79B2*; **B,** *CYP79B3*; **C,** *CYP71A13*; **D,** *PAD3*; **E,** *CYP83B1*; **F,** *SUR1*; and **G,** *WRKY33* by *Piriformospora indica* in *Arabidopsis* roots. RNA was isolated from the roots of wild-type (WT, Col 0) seedlings and different mutants as indicated which were either co-cultivated with *P. indica* for 14 days or mock treated. After real-time polymerase chain reaction analysis, the $mRNA_{+P.\ indica}/mRNA_{mock\text{-}treated}$ ratio was calculated. Values are based on five independent biological experiments. Error bars were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors.

action between the endophytic fungus *P. indica* and *Arabidopsis* requires a balanced activation of defense processes, and mutants defective in specific defense-related genes are often unable to restrict fungal growth (Camehl and Oelmüller 2010; Camehl et al. 2010; Johnson and Oelmüller 2009; Sherameti et al. 2008). Here, we demonstrate that IAOx-derived indole compounds are crucial for the beneficial interaction between the two symbionts, and that these compounds restrict root colonization. We also demonstrate that *P. indica*-induced cytoplasmic calcium elevation and MPK3 but not H_2O_2, RHD2 and RHD6, and the PDK1/OXI1 pathway are required for the activation.

RESULTS

Role of CYP79B2, CYP79B3, PAD3, and camalexin in the interaction between *P. indica* and *Arabidopsis* seedlings.

Co-cultivation of *Arabidopsis* roots with *P. indica* on agar plates for 14 days resulted in a low but significant (*t* test, $n = 5$, $P = 0.025$) induction of camalexin (127 ± 63 ng g^{-1} fresh weight) compared with the mock-treated control (50 ± 10 ng g^{-1} fresh weight), although it is synthesized in approximately two orders of magnitude lower concentrations compared with roots infected with pathogens such as *Plasmodiophora brassicae* (Siemens et al. 2008). Also, the mRNA levels for CYP79B2, CYP79B3, CYP71A13 (Nafisi et al. 2007), PAD3, and WRK33 (Qiu et al. 2008) are upregulated in colonized wild-type (WT) roots, whereas those for CYP83B1 and SUR1 are not (Fig. 1A to G). This demonstrates that the genes for the synthesis of IAOx-derived compounds, including camalexin but not I-GLS, are targets of signals from the fungus.

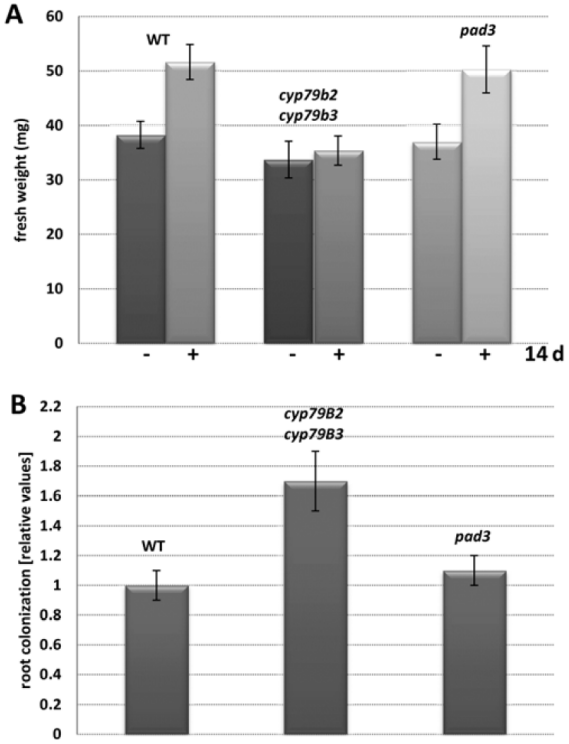


Fig. 2. A, Fresh weight of wild type (WT), *cyp79B2 cyp79B3*, and *pad3* seedlings after 14 days of co-cultivation with *Piriformospora indica* or mock treatment. **B,** Increase of root colonization based on quantitative reverse-transcription polymerase chain reaction of *cyp79B2 cyp79B3* and *pad3* seedlings with respect to WT after 14 days of co-cultivation with *P. indica*. Root colonization was calculated as outlined in the text. Here, mRNA-based data are shown; however, they are not significantly different from DNA-based data, which are not shown. The value for WT was set as 1.0 and the other values are expressed relative to it. Bars represent standard errors, based on six independent biological experiments with 12 seedlings per treatment per experiment. Relative errors of the proportion ($mRNA_{+P.\ indica}/mRNA_{mock\text{-}treated}$) in B are the sum of the individual relative errors.

In order to test whether IAOx-derived compounds are required for the beneficial interaction, we analyzed the growth response of *cyp79B2 cyp79B3* and *pad3* seedlings to *P. indica*. The weight of WT seedlings co-cultivated with *P. indica* on plant nutrient medium (PNM) plates for 14 days was approximately 35% higher compared with the mock-treated controls (Fig. 2A). The *pad3* seedlings also responded to the fungus and the fresh weight was promoted similar to WT. However, the weight of *P. indica*-colonized *cyp79B2 cyp79B3* seedlings was not significantly different from the mock-treated control (Fig. 2A). This demonstrates that camalexin synthesis is not essential for early processes during *P. indica*-mediated growth promotion and suggests that IAOx-derived compounds other than camalexin are required for the beneficial interaction.

Because camalexin exhibits cytotoxicity (Rogers et al. 1996), we tested whether it influences root colonization by *P. indica*. Therefore, the *P. indica translation elongation factor 1* (*Pitef1*) DNA and mRNA levels relative to the plant *glyceraldehyde-3-phosphate dehydrogenase C2* (*AtGAPC2*) DNA and mRNA levels were determined in colonized WT, *cyp79B2 cyp79B3*, and *pad3* plants. Colonization of the *cyp79B2 cyp79B3* but not *pad3* mutant roots was higher than that of WT roots (Fig. 2B). The data shown for *pad3* colonization are consistent with those published by Jacobs and associates (2011). Furthermore, they showed that *pen2-1* (defective in another enzyme linked to IAOx metabolism) displayed higher colonization. Because the *Pitef1*/*AtGAPC2* ratios were almost identical when the polymerase chain reactions (PCRs) were performed with DNA or with cDNA synthesized from mRNA of the colonized roots, the fungus is alive in the roots and expresses the *Pitef1* gene. We also stained fungal spores in the roots and found the same colonization pattern for the WT and the mutants (Fig. 3). Counting of the spores in a 1-cm segment (1 cm from root tip) confirmed the data from Figure 2B. Analysis of 120 seedlings showed that *cyp79B2 cyp79B3* roots (Fig. 3C and D) contain 59 ± 8 spores/cm, WT roots contain 39 ± 4 spores/cm, and *pad3* roots contain 37± 5 spores/cm. In all seedlings, spores are often associated with the root tips (Fig. 3B and D).

In colonized mutant roots, we observed a moderate but significant upregulation of plant defense genes (Fig. 4A to C), and this was more pronounced in *cyp79B2 cyp79B3* than in *pad3* plants. In particular, *PDF1.2* and *germin* were strongly upregulated in *cyp79B2 cyp79B3* roots (Fig. 4A and B). Comparison of Figures 2 and 4 demonstrates that higher root colonization rates correlate with the activation of defense genes and the loss of benefits for the plants. This suggests that metabolites synthesized by the CYP79B enzymes restrict growth of *P. indica*.

Cytoplasmic Ca^{2+} elevation and MPK3 but not RHD2, RHD6, PLDα1, PDK1, or OXI1 are involved in the *P. indica*-induced upregulation of the mRNAs for CYP79B2, CYP79B3, CYP71A13, PAD3, and WRKY33.

Camalexin is induced by many plant pathogens in the roots and shoots of *Arabidopsis*. *PAD3* activation requires MPK3 (Ren et al. 2008) and is stimulated by biotic and abiotic stresses leading to the formation of ROS (Van Breusegem et al. 2008). ROS production activates OXI1 which, in turn, is required for

full activation of MPK3 and MPK6 (Rentel et al. 2004). In addition to ROS, OXI1 is also activated by the PDK1, after binding to the second messenger phosphatidic acid (Camehl et al. 2011).

To identify upstream components in the signaling cascade leading to *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA induction by *P. indica* in *Arabidopsis* roots, mutants of MPK3, the ROS-producing RHD2, the phosphatidic-acid-producing phospholipase Dα1, the OXI-activating PDK1s, and ROS-activated OXI1 were compared with the WT after co-cultivation with *P. indica* (Fig. 1A to G). The *rhd6* mutant was included to investigate the role of root hairs for the induction of the genes by the fungus. Furthermore, we included three genetically distinct *Arabidopsis* ethyl-methane-sulfonate mutants, called *deficient in cytoplasmic calcium elevation* (*dce*)*1* to *dce3* which are impaired in inducing cytoplasmic Ca^{2+} elevation in response to the fungus and a fungal cell wall preparation (Fig. 5) (Vadassery et al. 2009). Because these mutants do not respond to the fungus in terms of growth promotion (*unpublished data*), the rapid increase in cytoplasmic Ca^{2+} elevation appears to be required for establishing the beneficial symbiosis.

PAD3 (Fig. 1D), *CYP79B2* (Fig. 1A), *CYP79B3* (Fig. 1B), *CYP71A13* (Fig. 1C), and *WRKY33* (Fig. 1G) were downregulated by *P. indica* in all mutants impaired in cytoplasmic Ca^{2+} elevation and in the *mpk3* mutant, compared with WT, *rhd2*, *rhd6*, the *pdk1.1 pdk1.2* double knock-out line, and *oxi1* mutants (Fig. 1A to D and G) but CYP83B1 (Fig. 1E) and SUR1 (Fig. 1F) were not affected. Although phosphatidic acid might be a second messenger in *P. indica*-mediated growth promotion (Camehl et al. 2011) and phospholipase Dα1 is a main producer of phosphatidic acid in *Arabidopsis* roots (Devaiah et al. 2006), the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13,* and *WRKY33* mRNA levels are not significantly different in a mutant lacking phospholipase Dα1 compared with the WT control (Fig. 1A to D and G). Furthermore, we could not detect an increase in the ROS level in infected WT and mutant roots (Camehl et al. 2011; Vadassery et al. 2009). This suggests that *P. indica* stimulates the expression of these genes through a Ca^{2+}- and MPK3-dependent pathway but does not require production of ROS through the NADH oxidase RHD2 and the activity of the PDK1 and OXI1 (discussed below).

PAD3 expression is induced locally by *P. indica*.

We analyzed the expression of a *PAD3 promoter::uidA* fusion in mock-treated and colonized transgenic *Arabidopsis* seedlings using the root system in split petri dishes. Stimulation of β-glucuronidase (GUS) was only visible in root sections surrounded by fungal mycelium (Fig. 6A). A lower level of GUS activity was found in mock-treated roots (Fig. 6A). In particular, *P. indica*-induced GUS activity accumulated mainly in lateral roots during the early phase of interaction but, later, we noticed GUS staining in the main roots. To test whether this reflects the regulation of mRNA levels for IAOx-derived compounds, we isolated RNA from the roots and from preparations enriched in lateral roots and root hairs (discussed below) from colonized and uncolonized areas of the seedlings. The RNA extracted from fungus-exposed root areas after 14 days of co-cultivation of the two symbionts contained approximately three times higher mRNA levels for the *P. indica*-inducible *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* genes compared with the root areas which were not exposed to fungal hyphae. When RNA was extracted from the fungus-exposed lateral root areas alone, we observed a much higher stimulation of these mRNA levels (Fig. 6B). Again, *CYP83B1* and *SUR1* were not or much less upregulated compared with the other genes. This suggests that the former genes are induced locally by the fungus, preferentially in lateral roots and root hairs. We also analyzed relative GUS activity in lateral roots

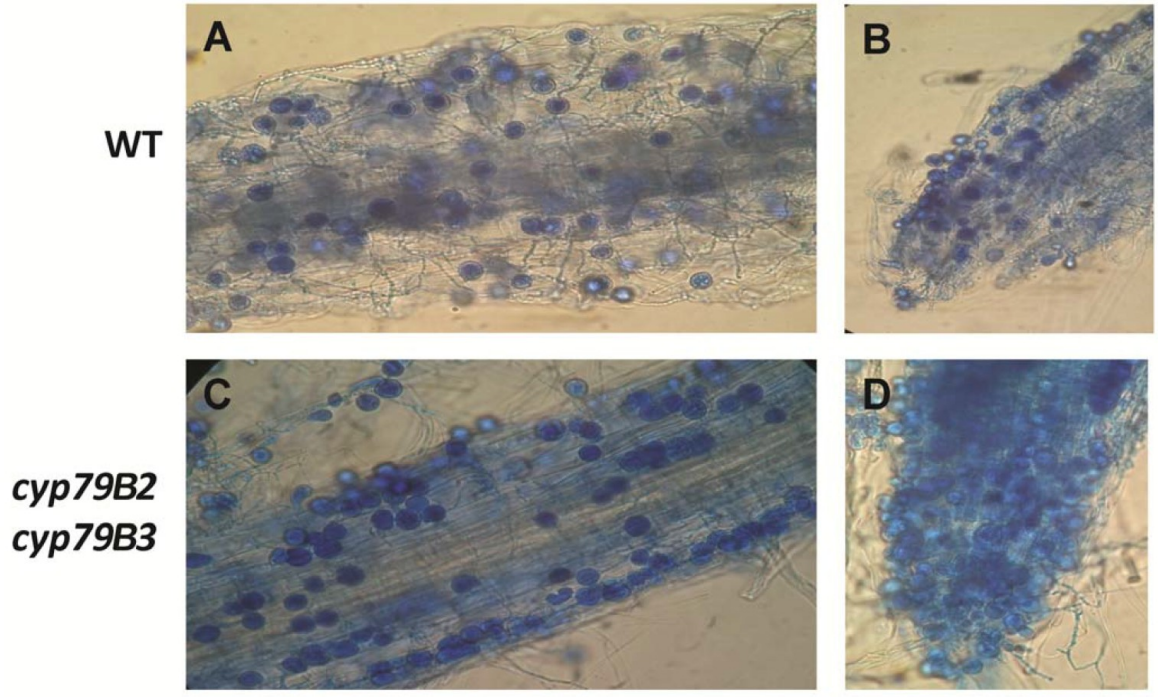


Fig. 3. Distribution of fungal spores in *Arabidopsis* **A** and **C,** root segments (1 to 2 cm away from the root tip) or **B** and **D,** at the root tip of **A** and **B,** the wild type (WT) and **C** and **D,** *cyp79B2 cyp79B3*. Representative pictures for seedlings after 14 days of co-cultivation with *Piriformospora indica* on PNM plates. The colonization pattern was the same for WT and mutant seedlings on PNM plates and in soil, except that the spore densities differed.

and root hairs at different time points after *P. indica* treatment (Fig. 6C). We noticed that, from 24 h onward, GUS expression was rapidly increased in *P. indica* co-cultivated lateral roots and root hairs (Fig. 6C).

Role of CYP79B2, CYP79B3, PAD3, and camalexin in the interaction between *P. indica* and *Arabidopsis* plants in soil.

After 14 days of co-cultivation on PNM medium (details below), colonized and mock-treated WT, *cyp79B2 cyp79B3*, and *pad3* seedlings were transferred to soil. After 42 days, *P. indica*-infected WT plants produced 32 ± 4% more shoot biomass than the mock-treated control. In contrast, the shoot biomass of infected *cyp79B2 cyp79B3* and *pad3* plants was reduced compared with the mock-treated control (Fig. 7A). Thus, long-term harmony between the two symbionts requires CYP79B2/CYP79B3 and PAD3.

After 42 days on soil (after 42 days, the vegetative phase was over, most of the plants flowered, and there was not any more camalexin increase), the camalexin level in the colonized WT roots was not higher than in the mock-treated control (0.75 ± 0.46 µg g^{-1} fresh weight; mock-treated control: 1.17 ± 0.58 µg g^{-1} fresh weight). However, when compared with seedlings, the camalexin levels in the roots of adult plants were five- to 10-fold higher. Thus, the overall amount of camalexin per fresh weight increased in the WT roots during the 42 days on soil but the small stimulatory effect of *P. indica* observed for the seedlings was no longer detectable for adult plants. Also the *PAD3*, *CYP79B2*, *CYP79B3*, *CYP71A13*, and *WRKY33* mRNA levels were no longer upregulated by the fungus in WT roots (Supplementary Table S2): in none of the experiments did we observe a significant stimulation by the fungus. Furthermore, pathogenesis-related (*PR*) genes (*PR-1*, *PDF1.2*, *phenylalanine ammonia lyase* [*PAL*], and *germin*) which are highly upregulated in colonized roots of *cyp79B2 cyp79B3* seedlings (Fig. 4A and B) were also upregulated in the roots of colonized adult *cyp79B2 cyp79B3* and *pad3* plants but at a lower scale (Fig. 7B1 and B2). The ROS-related *RHD2* and the root-hair-controlling *RHD6* genes are not regulated by *P. indica* (Fig. 7B3). We also could not detect ROS production in the roots of adult WT, *cyp79B2 cyp79B3*, and *pad3* plants (Supplementary Table S3). Taken together, defense processes in the colonized mutant and WT roots of adult plants are not much activated by the fungus compared with the situation in the seedlings.

However, root colonization was much higher in the mutant plants compared with the WT (Fig. 7C). After 42 days in soil, the *Pitef1* DNA or cDNA/*AtGAPC2* DNA or cDNA ratios for the *cyp79B2 cyp79B3* and *pad3* plants were 11 and 5 times higher, respectively, than the ratio for WT plants (Fig. 7C). Kinetic analysis demonstrates that these ratios increased gradually with longer co-cultivation times on soil (Supplementary Table S4). Microscopic inspection of the colonization did not discover significant differences to the colonization pattern found for seedlings (Fig. 3): younger root sections were more colonized than older regions of the roots and spores were mainly found in association with lateral roots. Thus, the low level of defense gene activation in combination with the absence of IAOx-derived indole compounds promotes fungal

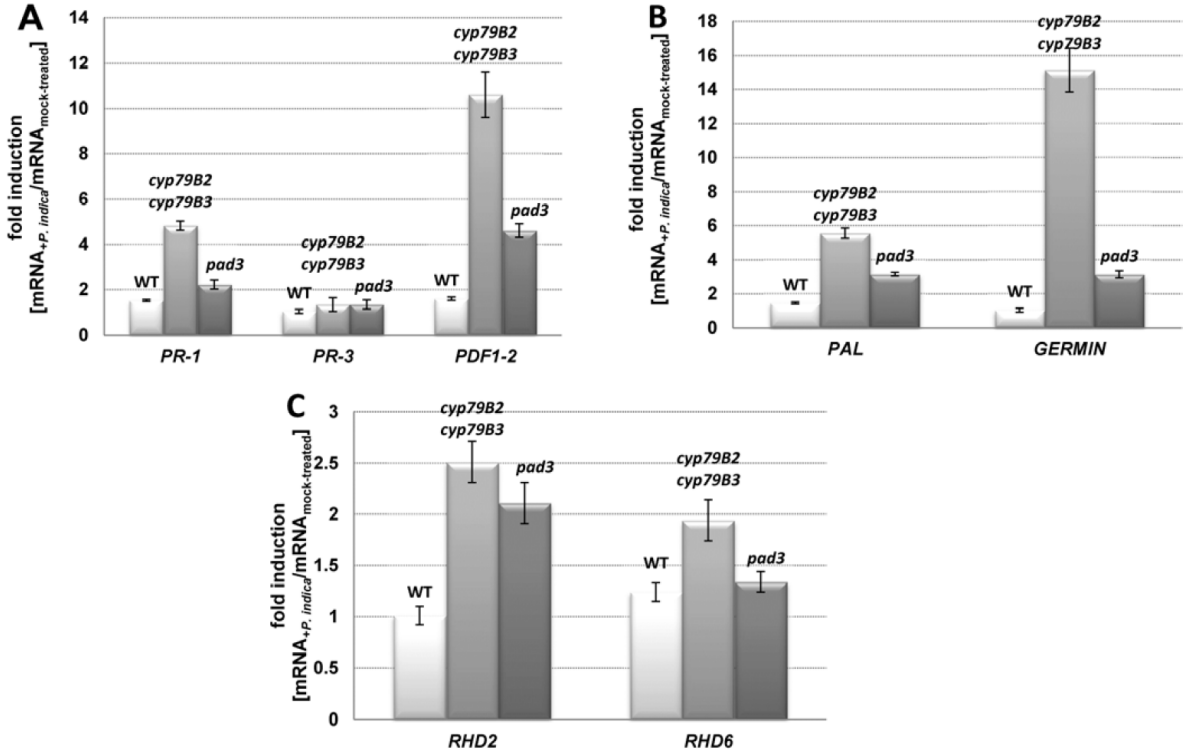


Fig. 4. Fold induction of the mRNA levels for pathogenesis-related (*PR*) genes **A,** *PR-1*, *PR-3*, and *PDF1.2*; **B,** phenylalanine ammonia lyase (*PAL*) and *germin*; and **C,** root-hair-deficient (*RHD*) genes *RHD2* and *RHD6* by *Piriformospora indica* in *Arabidopsis* roots. RNA was isolated from the roots of wild-type (WT) and mutant (*cyp79B2 cyp79B3* and *pad3*) seedlings which were either co-cultivated with *P. indica* for 14 days or mock treated. After real-time polymerase chain reaction analysis, the $mRNA_{+P.\ indica}/mRNA_{mock\text{-}treated}$ ratio was calculated. Data are based on five independent biological experiments with three replications. Error bars were calculated as standard errors. Relative errors of the proportion are the sum of the individual relative errors.

growth (discussed below). Overcolonization of the roots might be the reason for the reduced benefits for the plants. This confirms that *P. indica*-mediated long-term benefits for the plants require CYP79B2/CYP79B3 and PAD3.

DISCUSSION

Beneficial interaction between *P. indica* and *Arabidopsis* requires IAOx-derived compounds.

We demonstrated that IAOx-derived compounds are essential for initial steps in the beneficial interaction between *P. indica* and *Arabidopsis*, because seedlings of the *cyp79B2 cyp79B3* double mutants do not respond to the fungal infection with growth promotion (Fig. 2A). Because *pad3* seedlings respond to *P. indica* (Fig. 2A), camalexin seems not to be important during the initial phase of interaction. However, PAD3 is required during later stages (Fig. 7A).

Therefore, camalexin (Böttcher et al. 2009) and other IAOx-derived compounds may be involved in independent processes in this symbiosis. Similar observations have been reported in different pathosystems. Schlaeppi and associates (2010) showed that a deficiency in either camalexin or I-GLS accumulation had only a minor effect on the disease resistance of *Arabidopsis* against *Phytophthora brassicae* infection, while *cyp79B2 cyp79B3* was highly susceptible to *P. brassicae*. Sanchez-Vallet and associates (2010) have demonstrated that tryptophan-derived secondary metabolites may have differential contributions in non-host resistance to necrotrophic and biotrophic pathogens. Analysis of additional mutants impaired in the synthesis of specific IAOx-derived secondary metabolites will help to understand their role in this beneficial symbiosis.

The higher degree of fungal colonization in the roots of *cyp79B2 cyp79B3* seedlings (Fig. 2B) and adult *cyp79B2 cyp79B3* and *pad3* plants (Fig. 7C) and the initial activation of defense genes against the fungus in the roots of the *cyp79B2 cyp79B3* seedlings suggest that IAOx and its derived compounds, including camalexin, participate in the control of fungal growth. Initially, the roots responded to the lack of IAOx and its derived compounds by activating other defense genes such as *PR-1, PDF1.2, PAL*, and *germin* (Fig. 4A and B). However, this mechanism appears to be restricted to early phases of the symbiosis and is no longer active in adult plants (Fig. 7B1 and B2). In the roots of adult mutants, expression of these defense genes is much lower when compared with the expression in seedling's roots, either because the plant recognizes the microbe as a friendly symbiont and no longer activates defense genes or because the symbiont actively represses the host's defense response (Jacobs et al. 2011). The low level of defense might explain the high colonization rate and the loss of benefits for the adult mutants.

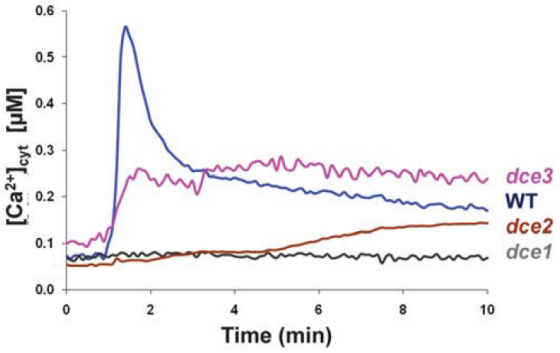


Fig. 5. *Piriformospora indica* cell wall extract (CWE) induces changes in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) levels in the roots of apoaequorin-transformed *Arabidopsis* wild-type (WT) seedlings or in the roots of three *Arabidopsis* mutants, *deficient in cytoplasmic calcium elevation* (*dce)1* to *dce3*. $[Ca^{2+}]_{cyt}$ elevation was induced by the application of 50 µl of CWE to *Arabidopsis* roots. The $[Ca^{2+}]_{cyt}$ was calculated from the relative light units (RLU) measured at 5-s integration times for 10 min. In all the experiments, water was used as control and gave background readings. The four calcium signatures are representative of several hundred measurements (Vadassery and associates 2009).

Although overall defense gene activation in WT and mutant roots by *P. indica* is relatively mild compared with pathogen infections, previous studies have demonstrated that it is required for the beneficial interaction. Disturbance of ethylene signaling (Camehl and Oelmüller 2010; Camehl et al. 2010) or manipulation of the abundance of the root myrosinase PYK10 (Sherameti et al. 2008) have severe consequences for the beneficial interaction and are associated with a partial shift from mutualism to parasitism. In agreement with these observations, a number of reports have demonstrated that mycorrhiza rely on a mild activation of defense responses (de Hoff et al. 2009; Fester and Hause 2005; Gutjahr and Paszkowski 2009; Herre et al. 2007; Martin et al. 2007; Pozo and Azcón-Aguilar 2007; Purin and Rillig 2008; Strack et al. 2003). IAOx and its derived compounds, including camalexin, may provide another example for a defense process that restricts growth of fungi in beneficial root symbioses.

In this context, comparison of the root colonization of the seedlings (Fig. 2B) and adult plants (Fig. 7C) is interesting. After 14 days of co-cultivation of the seedlings with the fungus on PNM plates, they were transferred to soil for an additional 42 days. Root colonization in adult WT roots was approximately 40% of that of WT seedlings (Figs. 2B and 7C) although, in both cases, the increase in fresh weight is >30% relative to the mock-treated controls (Figs. 2A and 7A). This suggests that the *P. indica*-mediated growth response of the WT seedlings could probably be achieved by even less colonization of roots. Furthermore, the root colonization of adult *cyp79B2 cyp79B3* plants is five times and that of adult *pad3* plants three times higher than that of the WT seedlings in plates. This indicates that the degree of root colonization fluctuates, which has also been observed for mycorrhizal fungi (Kennedy 2010). On the other hand, comparison of these values with the increase in biomass during the 42 days on soil clearly indicates that the fungus propagates in WT and mutant roots and that the propagation is much stronger in mutant than in WT roots. The higher degree of colonization of the fungus in adult mutant plants leads to reduction of biomass, where the balance of interaction is shifted to a low level of parasitism. Finally, the calculated *Piref1/AtGAPC2* ratios did not change significantly for seedlings and adult plants regardless of whether they are based on cDNA or DNA levels (Supplementary Fig. S1). This suggests that the fungus is viable and expresses the *Piref1* gene during growth on soil.

The split-root-system experiment in combination with the expression analysis of genes involved in the synthesis of IAOx and its derived products demonstrate that the *P. indica*-induced responses are local. The relative GUS activity was rapidly increased in *P. indica* co-cultivated lateral roots and root hairs (Fig. 6C) at an early phase of interaction (from 24 to 60 h). No stimulatory response has been observed in the uninfected sections of the roots and in mock-treatment. The mRNA levels were much higher in infected lateral roots when compared with the mock treatment (Fig. 6B). Also, Kliebenstein and associates (2005) and Schuhegger and associates (2007) reported that camalexin synthesis genes are typically upregulated locally in proximity to the sites of pathogen infections, and both biotrophic and necrotrophic pathogens can induce camalexin biosynthesis. The local camalexin accumulation corresponds to a

strong induction of tryptophan and camalexin biosynthetic genes (Schuhegger et al. 2006, 2007). It is believed that lesions of the infected tissue are a prerequisite for camalexin induction in nature, although an autoclaved yeast suspension (Raacke et al. 2006) and peptidoglucan preparations (Gust et al. 2007) can induce the phytoalexin synthesis in leaves without observed cell death. Deshmukh and associates (2006) and Jacobs and associates (2011) have demonstrated that root regions undergoing cell death are more colonized by *P. indica* than other regions. Therefore, the interaction occurs preferentially at sides with injured root cells. We observed that, other than *P. indica*, a cell wall preparation from the fungus that promotes plant growth (Vadassery et al. 2009) induces *PAD3, CYP79B2, CYP79B3, CYP71A13*, and *WRKY33* mRNA levels in a dose- and time-dependent manner in the roots of *Arabidopsis* seedlings (data not shown). Therefore, the response does not necessarily require local injuries of individual root cells (e.g., by hyphal penetration) but can also be induced by a *P. indica*-released microbe-associated molecular pattern (MAMP) that is present in the cell wall preparation. Our data suggest that the *P. indica*-induced response occur locally, although Truman and associates (2010) provided evidence for a role of indole-derived compounds in the establishment and maintenance of systemic immunity. Pathogens and pathogenic MAMPs often induce ROS production via NADH oxidases. Because ROS can function as a rapid, long-distance, auto-propagating signal (Mittler et al. 2011) and because *P. indica* and the *P. indica*-derived cell wall extract does not induce ROS production (Camehl et al. 2011; Vadassery et al. 2009), we may observe only a local induction of the *PAD3, CYP79B2, CYP79B3, CYP71A13*, and *WRKY33* mRNA levels.

Plant signaling events
for the *P. indica*-induced camalexin synthesis.

Defense gene activation in pathogenic plant–microbe interactions is often initiated by the activation of a plasma-membrane

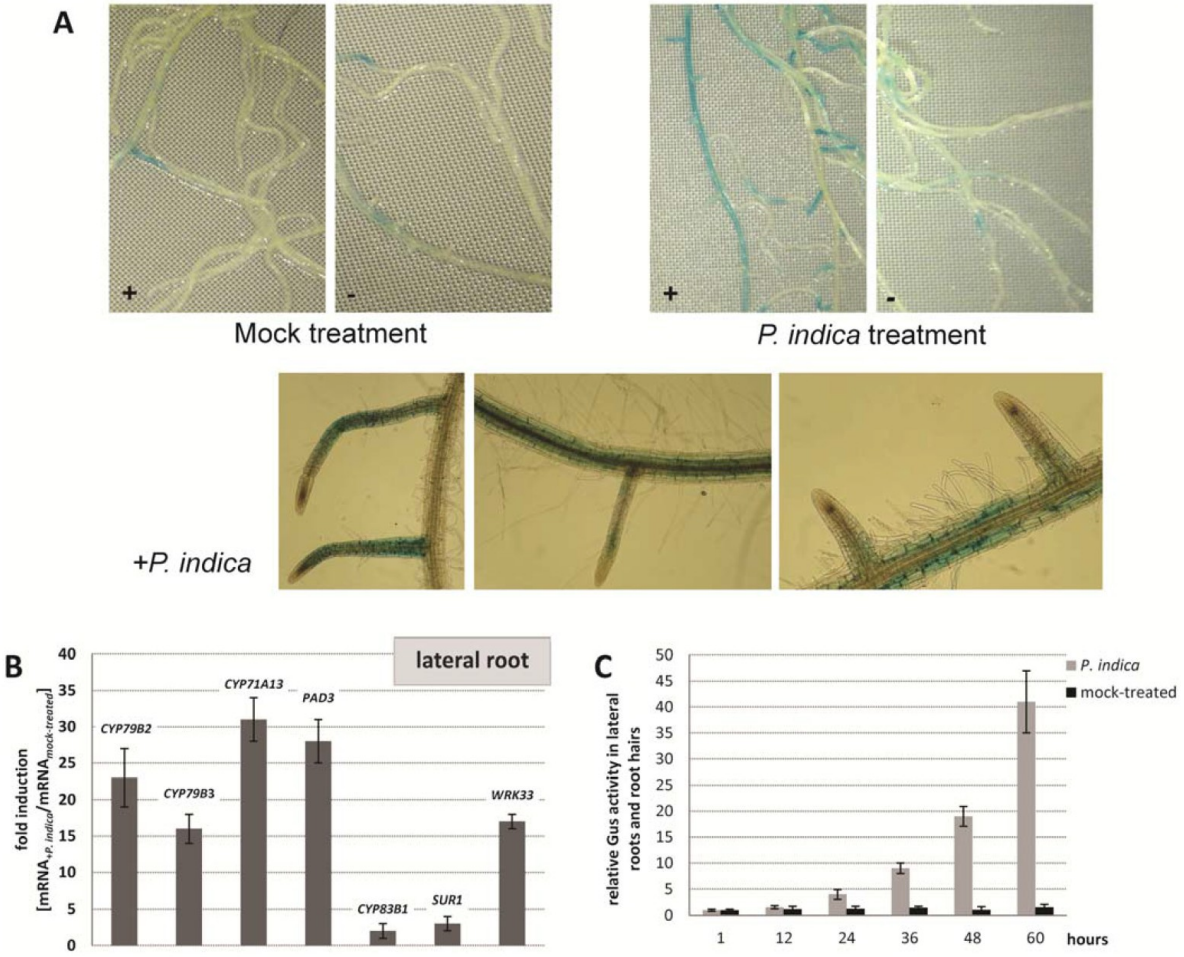


Fig. 6. A, β-Glucuronidase (GUS) staining of *pad3::uidA* roots in split petri dishes. Roots were not exposed (–) or exposed (+) to fungal hyphae. Mock treatment: without hyphae; *Piriformospora indica* treatment: plug was at the (+) site of the split petri dish. **A,** Bottom pictures show enlargement of *P. indica*–exposed root sections from split petri dishes. **B,** Fold induction of the mRNA levels for *CYP79B2, CYP79B3, CYP71A13, PAD3, CYP83B1, SUR1*, and *WRKY33* by *P. indica* in the lateral root and root hair preparation from *Arabidopsis*. RNA was isolated from the root material of wild-type (WT) seedlings which were either co-cultivated with *P. indica* for 14 days or mock treated. After real-time polymerase chain reaction analysis, the $mRNA_{+P.\ indica}$/$mRNA_{mock\text{-}treated}$ ratio was calculated. Data are based on four independent biological experiments with three replications. Error bars represent standard errors (SE). Relative errors of the proportion are the sum of the individual relative errors. **C,** Relative GUS activity in lateral roots and root hairs of mock-treated and *P. indica*-infected seedlings at the early phase of interaction (from 0 to 60 h) of WT roots. Data are based on four independent biological experiments with 10 replications. Error bars represent SE. Relative errors of the proportion are the sum of the individual relative errors.

localized receptor, followed by intracellular Ca^{2+} elevations, H_2O_2 production, and MPK3 or MPK6 phosphorylation. In pathogenic interactions, cytoplasmic Ca^{2+} elevation proceeds ROS production and MPK3 activation (Blume et al. 2000; Jabs et al. 1997; Kroj et al. 2003; Lee et al. 2004; Ligterink et al. 1997; Nürnberger et al. 1994; Zimmermann et al. 1997). Induction of the cytochrome P450 genes under study required MPK3 but appeared to be independent of H_2O_2-dependent processes leading to defense gene activation via the PDK1/OXI1 pathway (Fig. 1). Interestingly, three genetically distinct ethyl-methanesulfonate mutants that are impaired in inducing cytoplasmic Ca^{2+} elevation in response to *P. indica* and a cell wall extract from this fungus also fail to induce camalexin synthesis genes. Therefore, cytoplasmic Ca^{2+} elevation is required for *P. indica*-induced stimulation of the genes for the P450 enzymes and WRK33, similar to the signaling in pathosystems.

MPK3 inactivation prevents the induction of camalexin-synthesizing genes by *P. indica* almost completely (Fig. 1). This is in agreement with a previous observation that a cell-wall extract from the fungus phosphorylates MPK in a Ca^{2+}-dependent manner (Vadassery et al. 2009). In pathogen-induced camalexin synthesis in leaves, MPK3 has an overlapping function with MPK6 and both kinases are required for full induction of camalexin biosynthesis (Ren et al. 2008). For instance, both

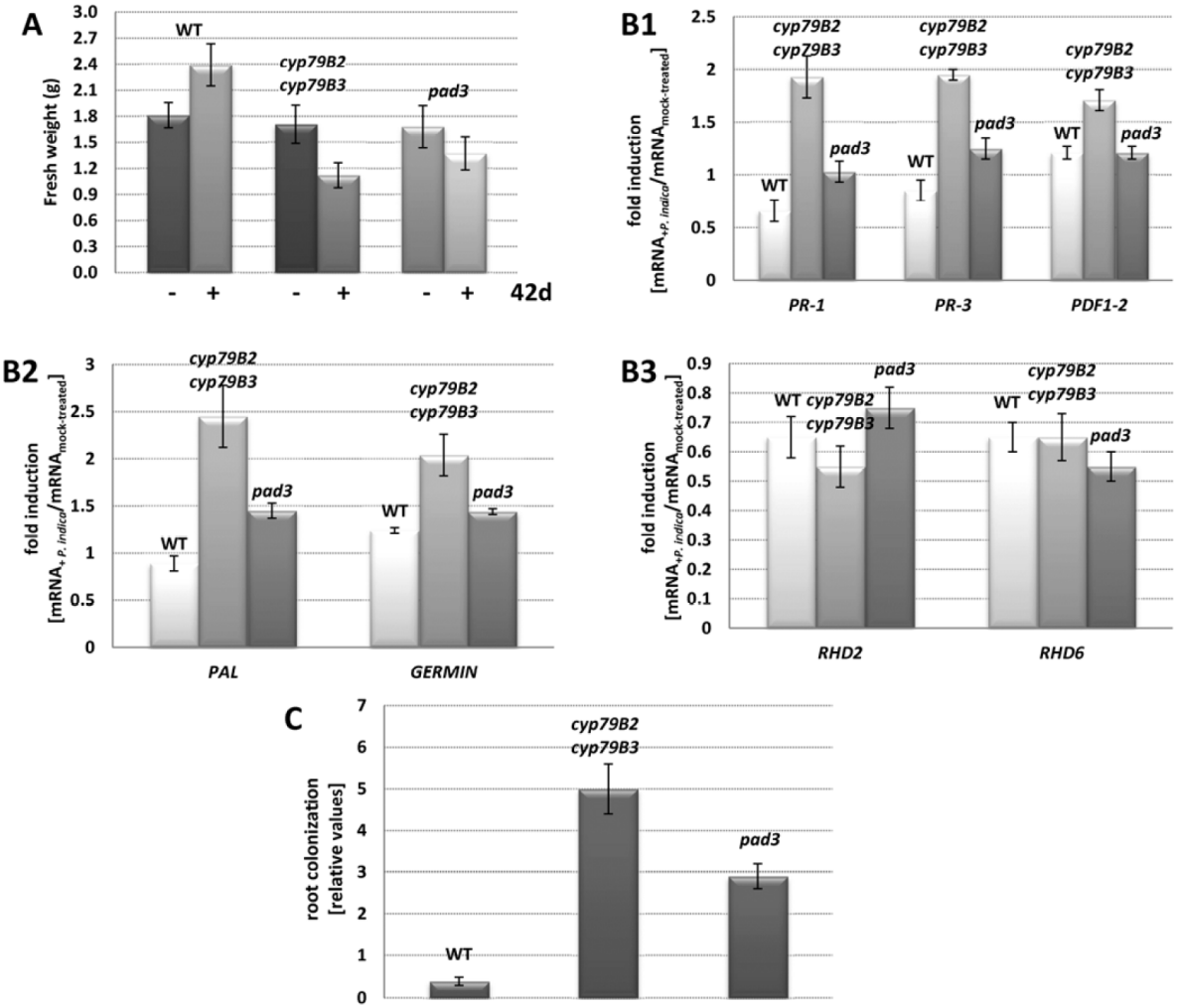


Fig. 7. A, Shoot fresh weight of the wild type (WT), *cyp79B2 cyp79B3*, and *pad3* of adult plants after 42 days in soil. WT and mutants seedlings were either co-cultivated with *Piriformospora indica* for 14 days or mock treated on PNM plates before transfer to soil. Standard errors (SE) are based on six independent biological experiments with 12 plants. **B,** Fold induction of the mRNA levels for pathogenesis-related (*PR*) genes **1,** *PR-1*, *PR-3*, and *PDF1.2*; **2,** phenylalanine ammonia lyase (*PAL*) and *germin*; and **3,** root-hair-deficient (*RHD*) genes *RHD2* and *RHD6* by *P. indica* in the roots of *Arabidopsis* WT, *cyp79B2 cyp79B3*, and *pad3* plants. RNA was isolated from the roots of WT and mutant seedlings which were either co-cultivated with *P. indica* for 14 days or mock treated on PNM plates before transfer to soil for 42 days. After real-time polymerase chain reaction analysis, the $mRNA_{+P.\ indica}$/$mRNA_{mock\text{-}treated}$ ratio was calculated. Data are based on six independent biological experiments with three replications. Error bars represent SE. Relative errors of the proportion are the sum of the individual relative errors. **C,** Root colonization (relative values) of WT, *cyp79B2 cyp79B3*, and *pad3* plants, which were either co-cultivated with *P. indica* for 14 days on PNM plates or mock treated before transfer to soil for 42 days. Root colonization was calculated as outlined in the text. Here mRNA-based data are shown; however, they are not significantly different from DNA-based data. To allow comparison of the root colonization data of the adult plants shown here with those of the seedlings shown in Figure 2B, the value for the WT control in this graph is expressed relative to that for WT seedlings in Figure 2B, which was set as 1.0. Bars represent SE, based on six independent biological experiments with three replications.

MPK3 and MPK6 are required for *Botrytis cinerea*-induced camalexin synthesis and subsequent limitation of fungal growth (Ren et al. 2008). Whether this is different for beneficial or root-specific fungi remains to be determined. *CYP79B2, CYP79B3*, and *PAD3* expression in response to *P. indica* is relatively low compared with the regulation by pathogens, which might be caused by the involvement of an additional control mechanism (for instance, active gene repression by either the plant or the fungus) to ensure a peaceful co-existence between the two symbionts.

Elevated H_2O_2 levels lead to the activation of OXI1, followed by MPK3 and MPK6 and PTI1-2 phosphorylation and transcriptional activation of defense genes (Anthony et al. 2004, 2006; Rentel et al. 2004). OXI1, in turn, is also activated by pathogens and MAMPs via phospholipid signaling and PDK1 activation (Anthony et al. 2006). Our data indicate that activation of *CYP79B2, CYP79B3, CYP71A13, PAD3*, and *WRKY33* by *P. indica* in roots is independent of H_2O_2 production, H_2O_2-initiated OXI1 signaling, and phosphatidic acid-activated PDK (Fig. 1). ROS is not produced in *P. indica*-colonized WT roots (Camehl et al. 2011; Vadassery et al. 2009). In agreement with this observation, RHD2, a major H_2O_2-producing NADH oxidase in roots, was not required for the activation of the P450 enzyme genes and *WRKY33* (Fig. 1). This is in agreement with previous observations that the PDK1-OXI1 cascade is important for the beneficial interaction between *P. indica* and *Arabidopsis* but not involved in defense gene activation (Camehl et al. 2011).

In summary, production of IAOx-derived metabolites is activated by *P. indica* during early phases of the interaction. This requires cytoplasmic Ca^{2+} elevation and MPK3; therefore, the fungus may utilize the classical defense pathway in the host. H_2O_2 production and activation of the H_2O_2-dependent OXI1 pathway is not involved in this response. After the beneficial symbiosis is established, defense processes, including the activation of genes for IAOx-derived metabolite synthesis, are no longer stimulated. However, for long-term harmony between the two symbionts, WT levels of IAOx-derived compounds are required to restrict root colonization.

MATERIALS AND METHODS

Growth conditions of plant and fungus.

Arabidopsis thaliana WT (ecotype Columbia-0) and mutant seed were surface-sterilized and placed on petri dishes containing Murashige-Skoog (MS) nutrient medium (Murashige and Skoog 1962). The *rhd2* line was obtained from V. Zarsky (Charles University, Prague); the *rhd6* (N6347), *pdk1.1* (N613251), and *pdk1.2* (N820699) lines from the Nottingham Arabidopsis Stock Centre; *mpk3* from D. Scheel (Halle); and *oxi1* from H. Hirt (Paris). The *pdk1.1 pdk1.2* double knock-out lines and all other mutants were described by Camehl and associates (2011). Homozygozity of the seed used for these studies was confirmed with gene-specific primers as previously published elsewhere. After cold treatment at 4°C for 48 h, plates were incubated for 10 days at 22°C under continuous illumination (100 µmol m^{-2} s^{-1}). *P. indica* was cultured as described previously on *Aspergillus* minimal medium (Hill and Kaefer 2001). More detailed information has been published by Johnson and associates (2011b). To ensure that the beneficial effect remain constant during fungal propagation, spores were reisolated from freshly infected *Arabidopsis* roots every 6 months for further propagation of the fungal hyphae (Johnson et al. 2011b, section A2).

Co-cultivation experiments and estimation of plant growth.

Twelve days after the growth of seedlings on MS media, *A. thaliana* seedlings of equal sizes were selected for co-cultivation experiments with or without *P. indica*. Co-cultivation was done as described by Johnson and associates (2011b, section C1, method 1). Because equal light intensity for each seedling is critical, it was checked weekly. Weights of seedlings were determined at 14 day after co-cultivation. For DNA and RNA isolation and gene expression studies, only roots were used.

Split root system, enrichment of lateral roots with root hairs.

Arabidopsis seedlings were grown on MS medium as described above. Then, plants were transferred on nylon membrane on split plates containing PNM medium. Half of the root system was posed to one-half of the plate with a fungal plug, while the other half was posed on other half of the plate. Mock treatment was done with a Kaefer medium plug. Both petri dishes without lids were kept in a larger petri dish for 14 days under the conditions described above to allow growth of the seedlings. Roots were harvested for RNA extraction. Alternatively, the two sections of the roots were cut separately from the aerial parts with a scalpel and dipped into liquid nitrogen for 1 s. Under this condition, smaller lateral roots and root hairs broke away from the main root and were recovered for RNA extraction.

Experiments on soil.

At 14 days after co-cultivation with *P. indica* or mock treatment on PNM plates, the seedlings were transferred to pots with uninfected soil for an additional 42 days (Johnson et al. 2011b, section C2, method 1). For harvest, the soil was removed from the roots with distilled water prior to RNA extraction. Plant were grown in a temperature (22°C)- and moisture-controlled room with light from the top (80 ± 10 µmol m^{-2} s^{-1} at the pot level) under short-day conditions (8 h of light and 16 h of darkness). The light intensity was monitored weekly.

Preparation of the cell wall extract from *P. indica*.

The cell wall extract was prepared using the protocol of Anderson-Prouty and Albersheim (1975), with modifications as described by Johnson and associates (2011a). Mycelia from 14-day-old liquid cultures were homogenized using mortar and pestle in water at 5 ml/g of mycelia. The homogenate was filtered using a coarse-sintered glass funnel. The residue was homogenized three times with water, twice with chloroform/methanol (1:1), and finally in acetone. This preparation was air dried for 2 h and the mycelial cell wall material was recovered. Elicitor fractions were prepared from mycelial cell walls by suspending 1 g in 100 ml of water and autoclaving for 30 min at 121°C. Autoclaving releases the active fraction. The suspension was centrifuged at 14,000 rpm for 10 min, was filter-sterilized using a 0.22-µM filter, and was concentrated to half. This fraction was further purified by passing it through a Reverse Phase Superclean LC-18 Cartridges (Sigma-Aldrich, Taufkirchen, Germany). The active fractions were collected and again concentrated to half (Johnson et al. 2011a).

Gene expression.

RNA was isolated from root tissue, including the lateral roots and the root hairs, and reverse-transcribed for real-time quantitative PCR analysis, using an iCycler iQ real-time PCR detection system and iCycler software (version 2.2; Bio-Rad, Munich). Total RNA was isolated from independent biological experiments of *Arabidopsis* roots. cDNA was synthesized using the Omniscript cDNA synthesis kit (Qiagen, Hilden, Germany) using 1 µg of RNA. For the amplification of the reverse-transcription PCR products, iQ SYBR Green Supermix (Bio-Rad) was used according to the manufacturer's protocol in a final volume of 25 µl. The iCycler was programmed to 95°C for 2 min;

40 cycles of 95°C for 30 s, 57°C for 40 s, and 72°C for 45 s; 72°C for 10 min; followed by a melting curve program of 55 to 95°C in increasing steps of 0.5°C. All reactions were performed in triplicate. The mRNA levels for each cDNA probe were normalized with respect to the *GAPC2* mRNA level. Fold-induction values of target genes were calculated with the ΔΔCP equation of Pfaffl (2001) and related to the mRNA level of target genes in mock-treated roots, which were defined as 1.0. Primer pairs with product sizes between 150 and 170 bp are given in Supplementary Table S1.

Root colonization.

Roots from plates or soil were harvested and roots from soil were washed intensively with distilled water before RNA or DNA extraction. *P. indica* was monitored with a primer pair for the elongation factor 1 (*Pitef1*) (Bütehorn et al. 2000). The *Pitef1* cDNA/DNA levels were expressed relative to the plant *GAPC2* cDNA/DNA levels. In order to ensure that the fungus was alive, the data presented in the manuscript were based on cDNA synthesized from RNA of the colonized roots. The same calculations were performed for PCR products obtained for fungal and root DNA. However, we did not observe significant differences between cDNA-based and DNA-based data. DNA isolation has been described previously (Camehl et al. 2011). Staining of hyphae and spores occurred with Trypan blue (0.05%) prior to light microscopy (Johnson et al. 2011b).

GUS assays for *pad3::uidA* lines.

Two independent *pad3* promoter*::uidA* lines were used for these experiments (Schuhegger et al. 2006). GUS staining was performed by transferring the nylon membrane with the seedlings (and hyphae) to a fresh petri dish prior to the application of the substrates, as described by Sherameti and associates (2002).

Camalexin determination.

The determination of camalexin concentrations has been described previously (Glawischnig et al. 2004). Five biological replicates were analyzed.

Ca^{2+} measurements, isolation of Ca^{2+} mutants.

Transgenic apoaequorin *A. thaliana* (Col-0) lines (Knight et al. 1997) were used for Ca^{2+} measurements. The roots were dissected from 14-day-old seedlings and reconstituted in 5 µM coelenterazine (P.J.K. GmbH, Kleinblittersdorf, Germany) in the dark overnight at 21°C. The luminescence counts obtained with a microplate luminometer (Luminoscan Ascent, version 2.4; Thermo Fisher Scientific, Schwerte, Germany) were calibrated using the equation by Rentel and Knight (2004) (Johnson et al. 2011a; Vadassery et al. 2009, appendix S1). The mutants were obtained after ethyl methanesulfonate mutagenesis. Approximately 450,000 individuals were screened with the cell wall extract from *P. indica* (J. M. Johnson and R. Oelmüller, *unpublished*). The three mutant lines used here were propagated on soil and M5 seed were used for these analyses. Crosses between the lines confirmed that they are not allelic.

Data analysis.

Data points represent the mean of six independent biological experiments with 12 to 24 seedlings or plants per treatment per experiment (if not otherwise stated). Samples were evaluated with a two-sample *t* test (+*P. indica* or mock treated) and analyses of variance (comparison of all data sets). Percent promotion is the proportion of x ($value_{+P.\ indica}$) and y ($value_{mock\text{-}treated}$), and the relative error of the proportion is the sum of the individual relative errors (standard errors of the mean).

ACKNOWLEDGMENTS

Work was supported by the Jena School of Microbial Communication and the Deutsche Forschungsgemeinschaft (OE133/28-1). We thank M. Hitzenbichler, S. Muβbach, and C. Röppischer for excellent technical assistance; and our colleges for the mutant and transgenic lines used for these experiments.

LITERATURE CITED

Anderson-Prouty, A. J., and Albersheim, P. 1975. Host–pathogen interactions. VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogens host. Plant Physiol. 56:286-291.

Anthony, R. G., Henriques, R., Helfer, A., Mészáros, T., Rios, G., Testerink, C., Munnik, T., Deák, M., Koncz, C., and Bögre, L. 2004. A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*. EMBO (Eur. Mol. Biol. Organ.) J. 23:572-581.

Anthony, R. G., Khan, S., Costa, J., Pais, M. S., and Bögre, L. 2006. The *Arabidopsis* protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. J. Biol. Chem. 281:37536-37546.

Bak, S., Tax, F. E., Feldmann, K. A., Galbraith, D. W., and Feyereisen, R. 2001. CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in *Arabidopsis*. Plant Cell 13:101-111.

Barlier, I., Kowalczyk, M., Marchant, A., Ljung, K., Bhalerao, R., Bennett, M., Sandberg, G., and Bellini, C. 2000. The SUR2 gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. Proc. Natl. Acad. Sci. U.S.A. 97:14819-14824.

Blume, B., Nürnberger, T., Nass, N., and Scheel, D. 2000. Receptor-mediated rise in cytoplasmic free calcium required for activation of pathogen defence in parsley. Plant Cell 12:1425-1440.

Böttcher, C., Westphal, L., Schmotz, C., Prade, E., Scheel, D., and Glawischnig, E. 2009. The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. Plant Cell 21:1830-1845.

Burow, M., Halkier, B. A., and Kliebenstein, D. J. 2010. Regulatory networks of glucosinolates shape *Arabidopsis thaliana* fitness. Curr. Opin. Plant Biol. 13:348-353.

Bütehorn, B., Rhody, D., and Franken, P. 2000. Isolation and characterization of Pitef1 encoding the translation elongation factor EF-1α of the root endophyte *Piriformospora indica*. Plant Biol. 2:687-692.

Camehl, I., and Oelmüller, R. 2010. Do ethylene response factors 9 and 14 repress *PR* gene expression in the interaction between *Piriformospora indica* and *Arabidopsis*? Plant Signal Behav. 9:5-8.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J., and Oelmüller, R. 2010. Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol. 185:1062-1073.

Camehl, I., Drzewiecki, C., Vadassery, J., Shahollari, B., Sherameti, I., Forzani, C., Munnik, T., Hirt, H., and Oelmüller, R. 2011. The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in *Arabidopsis*. PLoS Pathog. 7:e1002051.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Cortés-Penagos, C., and López-Bucio, J. 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant Physiol. 149:1579-1592.

Deak, M., Casamayor, A., Currie, R. A., Downes, C. P., and Alessi, D. R. 1999. Characterisation of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. FEBS (Fed. Eur. Biochem. Soc.) Lett. 451:220-226.

de Hoff, P. L., Brill, L. M., and Hirsch, A. M. 2009. Plant lectins: the ties that bind in root symbiosis and plant defence. Mol. Genet. Genomics 282:1-15.

Deshmukh, S., Hückelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F., and Kogel, K. H. 2006. . The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc. Natl. Acad. Sci. U.S.A. 103:18450-18457.

Devaiah, S. P., Roth, M. R., Baughman, E., Li, M., Tamura, P., Jeannotte, R., Welti, R., and Wang X. 2006. Quantitative profiling of polar glycerolipid species from organs of wild-type *Arabidopsis* and a phospholipase Dalpha1 knockout mutant. Phytochemistry 67:1907-1924.

Devarenne, T. P., Ekengren, S. K., Pedley, K. F., and Martin, G. B. 2006. Adi3 is a Pdk1 interacting AGC kinase that negatively regulates plant

cell death. EMBO (Eur. Mol. Biol. Organ.) J. 25:255-265.
Fester, T., and Hause, G. 2005. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza 15:373-379.
Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S., Brownlee, C., Jones, J. D., Davies, J. M., and Dolan, L. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422:442-446.
Geu-Flores, F., Møldrup, M. E., Böttcher, C., Olsen, C. E., Scheel, D., and Halkier, .BA. 2011. Cytosolic γ-glutamyl peptidases process glutathione conjugates in the biosynthesis of glucosinolates and camalexin in *Arabidopsis*. Plant Cell 23:2456-2469.
Glawischnig, E. 2007. Camalexin. Phytochemistry 68:401-406.
Glawischnig, E., Hansen, B. G., Olsen, C. E., and Halkier, B. A. 2004. Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 101:8245-8250.
Gust, A. A., Biswas, R., Lenz, H. D., Rauhut, T., Ranf, S., Kemmerling, B., Götz, F., Glawischnig, E., Lee, J., Felix, G., and Nürnberger, T. 2007. Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. J. Biol. Chem. 282:32338-32348.
Gutjahr, C., and Paszkowski, U. 2009. Weights in the balance: jasmonic acid and salicylic acid signaling in root-biotroph interactions. Mol. Plant-Microbe Interact. 22:763-772.
Hansen, C. H., Du, L., Naur, P., Olsen, C. E., Axelsen, K. B., Hick, A. J., Pickett, J. A., and Halkier, B. A. 2001. CYP83b1 is the oxime-metabolizing enzyme in the glucosinolate pathway in *Arabidopsis*. J. Biol. Chem. 276:24790-24796.
Herre, E. A., Mejía, L. C., Kyllo, D. A., Rojas, E., Maynard, Z., Butler, A., and Van Bael, S. A. 2007. Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. Ecology 88:550-558.
Hill, T. W., and Kaefer, E. 2001. Improved protocols for *Aspergillus* medium: trace elements and minimum media salt stock solutions. Fungal Genet. Newl. 48:20-21.
Hirt, H., Garcia, A. V., and Oelmüller, R. 2011. AGC kinases in plant development and defense. Plant Sign. Behavior 6:1030-1033.
Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K., and Scheel, D. 1997. Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defence gene activation and phytoalexin synthesis in parsley. Proc. Natl. Acad. Sci. U.S.A. 94:4800-4805.
Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Likpa, V., Kogel, K. H., and Schäfer, P. 2011. Broad-spectrum suppression of innate immunity is required for colonization of *Arabidopsis* roots by the fungus *Piriformospora indica*. Plant Physiol. 156:726-740.
Johnson, J. M., and Oelmüller, R. 2009. Mutualism and parasitism: life in an unstable continuum. What can we learn from the mutualitic interaction between *Piriformospora indica* and *Arabidopsis thaliana*? Endocyt. Cell Res. 19:81-111.
Johnson, J. M., Nongbri, P. L., Sherameti, I., and Oelmüller, R. 2011a. Calcium signaling and cytosolic calcium measurements in plants. Endocyt. Cell Res. 21:64-76. http://zs.thulb.uni-jena.de/receive/jportal_jparticle_00231945. Published online.
Johnson, J. M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Lou, B., Varma, A., and Oelmüller, R. 2011b. Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation—A model system to study plant beneficial traits. Endocyt. Cell Res. 21:101-113. http://zs.thulb.uni-jena.de/receive/jportal_jparticle_00247947. Published online.
Kennedy, P. 2010. Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. New Phytol. 187:895-910.
Kliebenstein, D. J., Rowe, H. C., and Denby, K. J. 2005. Secondary metabolites influence *Arabidopsis*/*Botrytis* interactions: variation in host production and pathogen sensitivity. Plant J. 44:25-36.
Knight, H., Trewavas, A. J., and Knight, M. R. 1997. Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J. 12:1067-1078.
Kroj, T., Rudd, J. J., Nürnberger, T., Gabler, Y., Lee, J., and Scheel, D. 2003. Mitogen-activated protein kinases play an essential role in oxidative burst-independent expression of pathogenesis-related genes in parsley. J. Biol. Chem. 278:2256-2264.
Lee, J., Rudd, J. J., Macioszek, V. K., and Scheel, D. 2004. Dynamic changes in the localization of MAPK cascade components controlling pathogenesis-related (PR) gene expression during innate immunity in parsley. J. Biol. Chem. 279:22440-22448.
Ligterink, W., Kroj, T., zur Nieden, U., Hirt, H., and Scheel, D. 1997. Receptor-mediated activation of a MAP kinase in pathogen defence of plants. Science 276:2054-2057.
Ljung, K., Hull, A. K., Celenza, J., Yamada, M., Estelle, M., Normanly, J., and Sandberg, G. 2005. Sites and regulation of auxin biosynthesis in *Arabidopsis* roots. Plant Cell 17:1090-1104.
Martin, F., Kohler, A., and Duplessis, S. 2007. Living in harmony in the wood underground: ectomycorrhizal genomics. Curr. Opin. Plant Biol. 10:204-210.
Masucci, J. D., and Schiefelbein, J. W. 1994. The *rhd6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin- and ethylene-associated process. Plant Physiol. 106:1335-1346.
Matsui, H., Miyao, A, Takahashi, A., and Hirochika, H. 2010. Pdk1 kinase regulates basal disease resistance through the OsOxi1–OsPti1a phosphorylation cascade in rice. Plant Cell Physiol. 51:2082-2091.
Mikkelsen, M. D., Fuller, V. L., Hansen, B. G., Nafisi, M., Olsen, C. E., Nielsen, H. B., and Halkier, B. A. 2009. Controlled indole-3-acetaldoxime production through ethanol-induced expression of CYP79B2. Planta 229:1209-1217.
Millet, Y. A., Danna, C. H., Clay, N. K., Songnuan, W., Simon, M. D., Werck-Reichhart, D., and Ausubel, F. 2010. Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. Plant Cell 22:973-990.
Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V., and Van Breusegem, F. 2011. ROS signaling: the new wave? Trends Plant Sci. 16:300-309.
Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
Nafisi, M., Sønderby, I. E., Hansen, B. G., Geu-Flores, F., Nour-Eldin, H. H., Nørholm, M. H. H., Jensen, N. B., Li, J., and Halkier, B. A. 2006. Cytochromes P450 in the biosynthesis of glucosinolates and indole alkaloids. Phytochem. Rev. 5:331-346.
Nafisi, M., Goregaoker, S., Botanga, C. J., Glawischnig, E., Olsen, C. E., Halkier, B. A., and Glazebrook, J. 2007. *Arabidopsis* cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. Plant Cell 19:2039-2052.
Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W. R., Hahlbrock, K., and Scheel, D. 1994. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. Cell 78:449-460.
Oelmüller, R., Sherameti, S., Tripathi, S., and Varma, V. 2009. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1-17.
Parisy, V., Poinssot, B., Owsianowski, L., Buchala, A., Glazebrook, J., and Mauch, F. 2007. Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. Plant J. 49:159-172.
Peškan-Berghöfer, T., Shahollari, B., Giong, P. H., Hehl, S., Markert, C., Blanke, V., Varma, A. K., and Oelmüller, R. 2004. Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. Physiol. Plant. 122:465-477.
Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:45.
Pozo, M. J., and Azcón-Aguilar, C. 2007. Unraveling mycorrhiza-induced resistance. Curr. Opin. Plant Biol. 10:393-398.
Purin, S., and Rillig, M. C. 2008. Parasitism of arbuscular mycorrhizal fungi: reviewing the evidence. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 279:8-14.
Qiu, J. L., Fiil, B. K., Petersen, K., Nielsen, H. B., Botanga, C. J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M. C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K. D., Mattsson, O., Glazebrook, J., Mundy, J., and Petersen, M. 2008. *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO (Eur. Mol. Biol. Organ.) J. 27:2214-2221.
Qutob, D., Kemmerling, B., Brunner, F., Küfner, I., Engelhardt, S., Gust, A. A., Luberacki, B., Seitz, H. U., Stahl, D., Rauhut, T., Glawischnig, E., Schween, G., Lacombe, B., Watanabe, N., Lam, E., Schlichting, R., Scheel, D., Nau, K., Dodt, G., Hubert, D., Gijzen, M., and Nürnberger, T. 2006. Phytotoxicity and innate immune responses induced by Nep1-like proteins. Plant Cell 18:3721-3744.
Raacke, I. C., Rad, U. v., Mueller, M. J., and Berger, S. 2006. Yeast increases resistance in *Arabidopsis* against *Pseudomonas syringae* and *Botrytis cinerea* by salicylic acid-dependent as well as -independent mechanisms. Mol. Plant-Microbe Interact. 19:1138-1146.
Rauhut, T., and Glawischnig, E. 2009. Evolution of camalexin and structurally related indolic compounds. Phytochemistry 70:1638-1644.
Rauhut, T., Luberacki, B., Seitz, H. U., and Glawischnig, E. 2009. Inducible expression of a Nep1-like protein serves as a model trigger system of camalexin biosynthesis. Phytochemistry 70:185-189.
Ren, D., Liu, Y., Yang, K. Y., Han, L., Mao, G., Glazebrook, J., and Zhang, S. 2008. A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 105:5638-5643.
Rentel, M. C., and Knight, M. R. 2004. Oxidative stress-induced calcium signalling in *Arabidopsis*. Plant Physiol. 135:1471-1479.

Rentel, M. C., Lecourieux, D., Ouaked, F., Usher, S. L., Petersen, L., Okamoto, H., Knight, H., Peck, S. C., Grierson, C. S., Hirt, H., and Knight, M. R. 2004. OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. Nature 427:858-861.

Rogers, E. E., Glazebrook, J., and Ausubel, F. M. 1996. Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*–pathogen interactions. Mol. Plant-Microbe Interact. 9:748-757.

Sanchez-Vallet, A., Ramos, B., Bednarek, P., López, G., Piślewska-Bednarek, M., Schulze-Lefert, P., and Molina, A. 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. Plant J. 63:115-127.

Schlaeppi, K., Abou-Mansour, E., Buchala, A., and Mauch F. 2010. Disease resistance of *Arabidopsis* to *Phytophthora brassicae* is established by the sequential action of indole glucosinolates and camalexin. Plant J. 62:840-851.

Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B. L., Olsen, C. E., Svatos, A., Halkier, B. A., and Glawischnig, E. 2006. CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. Plant Physiol. 141:1248-1254.

Schuhegger, R., Rauhut, T., and Glawischnig, E. 2007. Regulatory variability of camalexin biosynthesis. J. Plant Physiol. 164:636-644.

Sherameti, I., Nakamura, M., Yamamoto, Y. Y., Pfannschmidt, T., Obokata, J., and Oelmüller, R. 2002. Polyribosome loading of spinach mRNAs for photosystem I subunits is controlled by photosynthetic electron transport. Plant J. 32:631-639.

Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V. M., Nitz, I., Varma, A., Grundler, F. M., and Oelmüller, R. 2008. PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J. 54:428-439.

Siemens, J., Glawischnig, E., and Ludwig-Müller, J. 2008. Indole glucosinolates and camalexin do not influence the development of the clubroot disease in *Arabidopsis thaliana*. J. Phytopathol. 156:322-337.

Stone, J. M., Heard, J. E., Asai, T., and Ausubel, F. M. 2000. Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (fbr) *Arabidopsis* mutants. Plant Cell 12:1811-1822.

Strack, D., Fester, T., Hause, B., Schliemann, W., and Walter, M. H. 2003. Arbuscular mycorrhiza: biological, chemical, and molecular aspects. J. Chem. Ecol. 29:1955-1979.

Su, T., Xu, J., Li, Y., Lei, L., Zhao, L., Yang, H., Feng, J., Liu, G., and Ren, D. 2011. Glutathione-indole-3-acetonitrile is required for camalexin biosynthesis in *Arabidopsis thaliana*. Plant Cell 23:364-380.

Sugawara, S., Hishiyama, S., Jikumaru, Y., Hanada, A., Nishimura, T., Koshiba, T., Zhao, Y., Kamiya, Y., and Kasahara, H. 2009. Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 106:5430-5435.

Truman, W. M., Bennett, M. H., Turnbull, C. G., and Grant, M. R. 2010. *Arabidopsis* auxin mutants are compromised in systemic acquired resistance and exhibit aberrant accumulation of various indolic compounds. Plant Physiol. 152:1562-1573.

Vadassery, J., Ranf, S., Drzewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J., and Oelmüller, R. 2009. A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J. 59:193-206.

Van Breusegem, F., Bailey-Serres, J., and Mittler, R. 2008. Unraveling the tapestry of networks involving reactive oxygen species in plants. Plant Physiol. 147:978-984.

van der Luit, A. H., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T., and Munnik, T. 2000. Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. Plant Physiol.123:1507-1516.

Yamaguchi, T., Minami, E., Ueki, J., and Shibuya, N. 2005. Elicitor-induced activation of phospholipases plays an important role for the induction of defense responses in suspension-cultured rice cells. Plant Cell Physiol. 46:579-587.

Zhao, Y., Hull, A. K., Gupta, N. R., Goss, K. A., Alonso, J., Ecker, J. R., Normanly, J., Chory, J., and Celenza, J. L. 2002. Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev. 16:3100-3112.

Zimmermann, S., Nürnberger, T., Frachisse, J. M., Wirtz, W., Guern, J., Hedrich, R., and Scheel, D. 1997. Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defence. Proc. Natl. Acad. Sci. U.S.A. 94:2751-2755.

4.3 Manuscript III

Agony to Harmony - What decides? Calcium signaling in beneficial and pathogenic plant-fungus interactions - What can we learn from the *Arabidopsis/Piriformospora indica* symbiosis?

Joy Michal Johnson and Ralf Oelmüller

In *Molecular Microbial Ecology of the Rhizosphere (2013), Volume 2, pp 833-850,* First Edition. Edited by Frans J. de Bruijn.

Chapter 79

Agony to Harmony—What Decides? Calcium Signaling in Beneficial and Pathogenic Plant–Fungus Interactions—What Can We Learn from the *Arabidopsis/Piriformospora indica* Symbiosis?

Joy Michal Johnson and Ralf Oelmüller
Institute of General Botany and Plant Physiology, Friedrich-Schiller-University Jena, Germany

79.1 INTRODUCTION

Plant cells have evolved highly efficient mechanisms to perceive, transduce, and respond to a wide variety of internal and external signals. All living cells must signal continuously to adapt to their changing biotic and abiotic environments, and signaling requires messengers whose concentration varies with time and space (Clapham, 2007). Perception of signals through receptors often results in generation of nonproteineous messengers, which convey signals to the cellular machinery to initiate specific signaling events (Reddy and Reddy, 2004). A vast majority of these signaling events are accompanied by ionic changes (Takahashi et al., 1999). Evolution has adopted the positively charged Ca^{2+} ion and the negatively charged phosphate ion as the two primary signaling elements of cells (Clapham, 2007). The ability to alter local electrostatic fields and protein properties makes both Ca^{2+} and phosphate ions the two universal messengers of signal transduction. Ca^{2+}-binding and phosphorylation of proteins impart changes in positive and negative charges, respectively, thereby altering protein conformations, their interactions, and their subsequent biological functions (Westheimer, 1987).

In the course of evolution, Ca^{2+} has emerged as a versatile intracellular messenger and Ca^{2+} ions are involved in numerous plant signaling pathways (Sanders et al., 2002). Ca^{2+} signaling affects almost every aspect of a cell's life and death as it is a core transducer and regulator in sophisticated networks of signaling pathways (Sanders et al., 2002). Cells tightly control Ca^{2+} levels not only due to the nature of Ca^{2+} signaling, but also due to its cytotoxic effect. As the most tightly regulated ion within membrane-surrounded compartments and because of its flexibility in exhibiting different complex geometries, Ca^{2+} can easily form thousands of complexes with proteins, membranes, and organic acids (Kudla et al., 2010; Clapham, 2007). This feature, on the one hand, renders Ca^{2+} a toxic cellular compound at higher concentrations because it readily forms insoluble complexes, for example, with phosphate, but on the other hand, the required tight spatial and

Molecular Microbial Ecology of the Rhizosphere, Volume 2, First Edition. Edited by Frans J. de Bruijn.

temporal control of cellular Ca^{2+} concentration may have paved the way for the evolutionary emergence of Ca^{2+} signaling (Kudla et al., 2010). Under resting conditions, the cytoplasmic Ca^{2+} concentration ($(Ca^{2+})_{cyt}$) is maintained below 100 nm (Bush, 1995), 10^4 times less than that in the apoplastic fluid and 10^4 to 10^5 less than that in cellular organelles (Lecourieux et al., 2006). $(Ca^{2+})_{cyt}$ is rigorously regulated by the coordination of passive fluxes (Ca^{2+} channels) and active transport (Ca^{2+}-ATPases and Ca^{2+}-antiporters) across the plasma membrane and/or endomembranes of the internal Ca^{2+} stores, and the buffering capacity of the cytosol (Sanders et al., 2002; 1999; Bush, 1995).

Plants use different signaling pathways to respond to various external stimuli. The adaptation of plants to frequent environmental changes requires their ability to sense and to respond rapidly to these stimuli. Plants have to discriminate both the nature and the strength of these stimuli to mount an appropriate adaptive response for their survival (Mithöfer and Mazars, 2002). An important factor in plant survival is the early detection of and rapid response to a specific stimulus (Dodd et al., 2010; Kudla et al., 2010; Lecourieux et al., 2006; Hetherington and Brownlee, 2004; White and Broadley, 2003; Sanders et al., 2002; 1999). Most environmental challenges faced by plants, including biotic and abiotic stimuli, have been shown to induce changes in $(Ca^{2+})_{cyt}$ in the cell. Variations in the concentration of $(Ca^{2+})_{cyt}$ induce a large array of signals, which finally lead to different cellular responses (Sanders et al., 2002). Biotic agents or abiotic stimuli binding to their putative plasma membrane receptors provoke a large transient and sustained $(Ca^{2+})_{cyt}$ influx (cf. Dodd et al., 2010; Kudla et al., 2010; Lecourieux et al., 2006). Specificity in the Ca^{2+}-signaling system results from a multifactorial decision process, which includes a specific receptor, a unique Ca^{2+} signature, availability of a specific set of Ca^{2+} sensors, and their target proteins. Intracellular release of Ca^{2+} is one of the earliest events in signal perception. Increasing interest has been focused on the measurement of $(Ca^{2+})_{cyt}$ in the cell/tissue/organ/organism without destruction. Many Ca^{2+} signaling studies in plant cells are performed using the recombinant aequorin technology based on bioluminescence. Here we discuss different methods generally used to measure $(Ca^{2+})_{cyt}$ qualitatively and/or quantitatively with their advantages and disadvantages. As our laboratory is using the aequorin technology to measure $(Ca^{2+})_{cyt}$ in the model system *Arabidopsis thaliana* and a plant growth-promoting endophytic fungus, *Piriformospora indica*, more emphasis is placed on this technique.

79.2 BIOLOGICAL FUNCTIONS OF Ca^{2+} SIGNALING

Transient elevation in $(Ca^{2+})_{cyt}$ regulates processes in response to various abiotic and biotic stimuli, light, circadian rhythm, high and low temperature, touch, salt and drought, osmotic stress, hormones, fungal elicitors, nodulation and myc factors, microbes, and pathogens (reviewed by Lecourieux et al., 2006; Reddy and Reddy, 2004; White and Broadley, 2003; Rudd and Franklin-Tong, 2001; Sanders et al., 1999). Primary and autonomous Ca^{2+} responses have been confirmed for stomatal opening and closure in guard cells, growing pollen tubes, and root hairs. Ca^{2+} also participates in self-incompatibility during fertilization, resistance/susceptibility to pathogenic microbes, establishment of symbiosis in root hairs, and development of tip-growing structures such as pollen tubes and root hairs (cf. Table 79.1).

79.3 THE COMPONENTS OF Ca^{2+} SIGNALS/THE CENTRAL DOGMA OF Ca^{2+} SIGNALING

Ca^{2+} signaling is composed of a receptor, a system for generating the transient or slow increase in $(Ca^{2+})_{cyt}$ in response to a stimulus, recognition of the Ca^{2+}-signature by sensors, transduction of the signature message to targets, and other cellular systems responsible for returning $(Ca^{2+})_{cyt}$ to its prestimulus level (Hetherington and Brownlee, 2004; Reddy and Reddy, 2004). To act as an effective signal, $(Ca^{2+})_{cyt}$ needs to be maintained at sufficiently low levels in the cytoplasm to allow even small changes to bring about significant responses. Adenosine triphosphate (ATP)-fueled pumps (Ca^{2+}-ATPases) and transporters driven by the electrochemical gradients of other ions, particularly H^+ (H^+/ Ca^{2+} exchangers), play critical housekeeping roles in maintaining low resting Ca^{2+} in the unstimulated cell (White and Broadley, 2003).

The cell has multiple mechanisms for generating spatiotemporal patterns of Ca^{2+} elevation. Ca^{2+}-permeable channels are the key entry points for Ca^{2+} into the cytosol (Sanders et al., 2002). In plants, increases in $(Ca^{2+})_{cyt}$ arise from the influx of Ca^{2+} into the cytosol either from the apoplast or from internal stores, in which the Ca^{2+} concentration can be much higher compared to the cytosol, and/or from decreased efflux of Ca^{2+} from the cytosol (Hetherington and Brownlee, 2004). The Ca^{2+} concentration in resting cells is below 100 nM whereas it is in the millimolar range (1–10 mM) in extracellular and intracellular Ca^{2+} stores (Reddy, 2001;

Table 79.1 Different physiological processes initiated by changes in $(Ca^{2+})_{cyt}$ due to various stimuli (cf. White and Broadley, 2003)

Stimulus	$(Ca^{2+})_{cyt}$ Response	Cellular/physiological Process	Selected References
Abscisic acid	Elevated $(Ca^{2+})_{cyt}$ at cell periphery	Stomatal opening and closure	Klüsener et al. (2002), Allen et al. (1999)
Sphingosine-1-phosphate	Elevated $(Ca^{2+})_{cyt}$ around vacuole	Stomatal opening and closure	Ng et al. (2001)
Hydrogen peroxide	Oscillations in $(Ca^{2+})_{cyt}$	Stomatal opening and closure	Lecourieux et al. (2002)
Carbon dioxide	Elevated $(Ca^{2+})_{cyt}$ in guard cells	Stomatal opening and closure	Webb et al. (1996)
Nitric oxide, Cold	Elevated $(Ca^{2+})_{cyt}$ in guard cells	Stomatal opening and closure	Neill et al. (2002)
Reactive oxygen species (ROS) & Oxidative stress	Sustained high apical $(Ca^{2+})_{cyt}$	Root hair elongation	Foreman et al. (2003), Kwak et al. (2003)
	Oscillations in apical $(Ca^{2+})_{cyt}$	Root cell elongation	Mori and Schroeder (2004)
Extracellular Ca^{2+} influx	Oscillation of high apical $(Ca^{2+})_{cyt}$	Pollen tube elongation	Rudd and Franklin-Tong (2001)
Inositol-3-phosphate	Intracellular $(Ca^{2+})_{cyt}$ wave	Pollen tube self-incompatibility	Rudd and Franklin-Tong (2001)
Giberrelins	Slow rise in $(Ca^{2+})_{cyt}$	Seed germination	Anil and Rao (2001), Bush (1995)
ROS, Oxidative stress	Slow, sustained $(Ca^{2+})_{cyt}$ elevation	Apoptosis	Levine et al. (1996)
Red light	Elevated $(Ca^{2+})_{cyt}$	Phototransduction	Malhó et al. (1998)
Blue light	Brief spike in $(Ca^{2+})_{cyt}$ in sec	Photomorphogenesis	Malhó et al. (1998)
Circadian rhythms	Circadian $(Ca^{2+})_{cyt}$ oscillation	Circadian clock adaptation	Johnson et al. (1995)
Auxin	Slow, prolonged $(Ca^{2+})_{cyt}$ increase, Oscillations in $(Ca^{2+})_{cyt}$	Cell division and elongation	Plieth (2005)
ROS, Oxidative stress	Sustained $(Ca^{2+})_{cyt}$ elevation	Senescence	Huang et al. (1997)
High temperature	Elevated $(Ca^{2+})_{cyt}$ sustained for 15–30 min	Heat shock tolerance	Malhó et al. (1998)
Low temperature	Single brief $(Ca^{2+})_{cyt}$ spike in sec , Oscillations in $(Ca^{2+})_{cyt}$	Cold shock	Knight et al. (1996), Plieth (2005)
Mannitol	Slow spike (duration of min), sustained $(Ca^{2+z})_{cyt}$ elevation for h	Drought/hyper osmotic stress, adaptation by proline synthesis	Knight et al. (1997), Pauly et al. (2001), Malho et al. (1998)
Sodium chloride	Slow spike for few min, Sustained $(Ca^{2+})_{cyt}$ elevation for h	Salinity	Knight et al. (1997), Pauly et al. (2001)
Movement, touch, wind	Single brief $(Ca^{2+})_{cyt}$ spike (sec) Tissue $(Ca^{2+})_{cyt}$ wave	Mechanical stimulation, bending growth retardation	Knight et al. (1991), Plieth (2005)

Rudd and Franklin-Tong, 2001). The $(Ca^{2+})_{cyt}$ levels can be elevated up to 3 μM depending on the signal and cell type. Ca^{2+} influx occurs through specific Ca^{2+}-permeable channels, classified as nonselective cation channels (NSCCs). They include cyclic nucleotide-gated channels (CNGCs, 41 members), glutamate receptor channels (GLR, 21 members), and two-pore Ca^{2+} channels (TPC) (Hetherington and Brownlee, 2004; Reddy and Reddy, 2004). Ca^{2+} channels in the plasma membrane are often regulated by depolarization or hyperpolarization in response to biotic and abiotic stimuli (Hetherington and Brownlee, 2004; Reddy and Reddy, 2004). H^+/Ca^{2+} antiporters and Ca^{2+}-ATPases act in the opposite way to Ca^{2+}-permeable channels by pumping cytoplasmic Ca^{2+} into the exterior and/or intracellular stores (Reddy and Reddy, 2004). Their activities terminate the Ca^{2+} responses and replenish the Ca^{2+} stores. These channels, pumps, and carriers establish a cellular Ca^{2+} homeostasis by regulating the movement of Ca^{2+} ions between subcellular compartments and between the cell and its extracellular environment (Mithöfer and Mazars, 2002; Sanders et al., 2002; White and Davenport, 2002; Takahashi et al., 1999).

Ca^{2+} increase in plant cells often occurs as repetitive oscillations or spiking where the frequency (period), amplitude (magnitude), and shape (e.g., sinusoidal and square-wave) of the Ca^{2+} increase are determined by the nature of the inducing stimulus. The temporal changes in $(Ca^{2+})_{cyt}$ enable the ion to encode stimulus-specific information and define the nature and magnitude of the response (McAinsh and Pittman, 2009a, 2009b; Allen et al., 2001; McAinsh et al., 1995). This heterogeneity of increases in $(Ca^{2+})_{cyt}$ in terms of duration, amplitude, frequency, and spatial distribution leads to develop the concept of "Ca^{2+} signatures" (Hetherington and Brownlee, 2004; Webb et al., 1996), which is perceived by distinct Ca^{2+}-binding proteins. They establish an additional level of regulation and specificity (Batistič and Kudla, 2004), by decoding the information into specific protein–protein interactions, phosphorylation cascades, or transcriptional responses (Finkler et al., 2007; Luan et al., 2002; Sanders et al., 2002). Furthermore, cellular transporters and enzymatic activities are targets of Ca^{2+}-binding proteins (Finkler et al., 2007; Reddy and Reddy, 2004). Therefore, the dynamic interplay among the stimulus, its Ca^{2+} signature, and Ca^{2+}-sensing proteins contributes to the generation of the specific cellular response. Molecular genetic, biochemical, and bioinformatic approaches mainly with *Arabidopsis* resulted in the identification of a large number of proteins involved in the Ca^{2+} signaling networks (Reddy and Reddy, 2004).

In plants, Ca^{2+}-binding proteins fall into two main classes: Ca^{2+}-sensor relays and Ca^{2+}-sensor responders (Reddy and Reddy, 2004; Luan et al., 2002; Sanders et al., 2002). Sensor relays include calmodulin (CaM), CaM-related proteins, and calcineurin B-like (CBL) proteins. They function through bimolecular interactions and undergo a conformational change induced by Ca^{2+} before interacting with target proteins, or they change the activity or structure of their target proteins. Sensor responders include Ca^{2+}-dependent protein kinases (CDPK). They function through intramolecular interactions and undergo a Ca^{2+}-induced conformational change that alters the protein's own activity or structure (Harper et al., 2004; Zhang and Lu, 2003; Harmon et al., 2000). These two modes of decoding Ca^{2+} signals are used extensively in plants to provide a series of regulatory modules (Lecourieux et al., 2006). The presence of precisely localized networks of these Ca^{2+} targets together with the Ca^{2+} channels are crucial for decoding specific $(Ca^{2+})_{cyt}$ increases. Thus, the specificity in the Ca^{2+}-signaling system results from a multifactorial decision process (Lecourieux et al., 2006).

79.4 MEASURING ALTERATIONS IN $(Ca^{2+})_{CYT}$

Numerous methods for analyzing $(Ca^{2+})_{cyt}$ have been established (Takahashi et al., 1999). Studies on the involvement of $(Ca^{2+})_{cyt}$ in signaling rely mostly on techniques where spatial fluctuations in the free concentration can be visualized and absolute concentrations of Ca^{2+} can be measured (Rudd and Franklin-Tong, 2001). This requires nondestructive means and incorporation of a Ca^{2+}detection system into the cytoplasm. Three methods are mainly used: Ca^{2+} sensitive fluorescent dyes; cameleon, which is a fusion protein comprising a cyan-emitting version of green fluorescent protein (GFP) linked to calmodulin, a calmodulin-binding peptide, and an enhanced yellow-emitting GFP; and aequorin, a Ca^{2+}-binding photoprotein (Rudd and Franklin-Tong, 2001).

79.4.1 Measuring and Imaging $(Ca^{2+})_{cyt}$ Using Ca^{2+}-Sensitive Dyes

Ca^{2+}-sensitive fluorescent dyes allow the study of the qualitative, quantitative, spatial, and temporal distribution of intracellular (or cytoplasmic) Ca^{2+} concentrations in living cells (Takahashi et al., 1999). However, introducing these dyes into plant cells often causes technical problems, primarily due to the permeability of the cell wall and the plasma membrane. The principle is that when these dyes bind Ca^{2+} there is an increase in fluorescence, which is detected qualitatively and quantitatively either photometrically or by using an appropriate microscope. Single-wavelength "nonratiometric" dyes

and dual-wavelength "ratiometric" dyes are generally distinguished. Fluorescent dyes together with laser confocal microscopy provide significant information with respect to changes in the spatial distribution of Ca^{2+} in living cells in response to different stimuli. Besides limitations in permeability, the use of Ca^{2+}-sensitive fluorescent probes has additional limitations such as the cellular buffering capacity, autofluorescence, photodamage, leakage, compartmentalization, binding to other ions and proteins, or toxicity (cf. Takahashi et al., 1999).

79.4.2 Measuring and Imaging $(Ca^{2+})_{cyt}$ Using Cameleon

Cameleon is a fusion protein comprising a cyan-emitting version of GFP linked to calmodulin, a calmodulin-binding peptide (M13) and an enhanced yellow-emitting GFP (Gadella et al., 1999; Miyawaki et al., 1999). When the calmodulin domain binds Ca^{2+}, it induces a conformational change that can be detected by altered fluorescence resonance energy transfer (FRET) between the cyan and yellow fluorescent protein domains. Ca^{2+} levels can therefore be determined by measuring the efficiency of FRET (Gadella et al., 1999; Miyawaki et al., 1999). The cameleon system can be stably introduced into plants via transformation, and Allen et al. (1999) were the first to measure and image changes in $(Ca^{2+})_{cyt}$ in plant cells. The cameleon system does not need reconstitution, generates sufficient light for single cell imaging, and can be genetically engineered. This cameleon protein can also be targeted to specific cell types or organelles, which allows imaging of both global and compartmentalized $(Ca^{2+})_{cyt}$ changes (Emmanouilidou et al., 1999; Miyawaki et al., 1997). It can potentially be fused to signal transduction or Ca^{2+}-regulatory elements for dissecting Ca^{2+} signaling cascades in plants (Rudd and Franklin-Tong, 2001). This technique also has disadvantages like the small dynamic range of fluorescence intensity, and the fact that fluorescence of GFP is partially pH sensitive (Takahashi et al., 1999).

79.4.3 Measuring $(Ca^{2+})_{cyt}$ Using Aequorin

Aequorin is a complex Ca^{2+}-activated/binding photoprotein (Allen et al., 1976). It consists of an apo-aequorin and a luminophore prosthetic group, coelenterazine, covalently linked to the apo-aequorin through a peroxide bond (Musicki et al., 1986). Within the protein there are three EF-hand Ca^{2+}-binding sites (Persechini et al., 1989), a "hydrophobic region" with a coelenterazine-binding site (Tsuji et al., 1986), and a binding site for molecular oxygen (Shimomura and Johnson, 1978). Hydrophobic coelenterazine is easily taken up by plants, fungi, and mammalian cells and diffuses through plant and fungal cell walls, as well as through plasma membranes. In nature, the jelly fish, *Aequorea victoria*, synthesizes apo-aequorin, which combines with the prosthetic group coelenterazine in the presence of molecular oxygen to form the functional aequorin (Musicki et al., 1986; Charbonneau et al., 1985). When Ca^{2+} is bound to the EF hands, the protein undergoes a conformational change and behaves as an oxygenase that converts coelentrazine into excited coelenteramide. The excited coelentramide relaxes to the ground state by the release of carbon dioxide with the concomitant emission of blue light (λ 469 nm) (Shimomura and Johnson, 1978). This emitted blue light can be detected by a luminometer, or can be imaged, whereas the light emission relates directly to the Ca^{2+} concentration. Aequorin is highly sensitive to Ca^{2+} and can potentially detect free Ca^{2+} levels from 10 nM up to 100 μM.

Aequorin can be stably introduced into plant cells by transformation techniques (Knight et al., 1991). By adding coelenterazine to the transgenic tissue and after an appropriate stimulus, enough luminescence is produced, which allows reliable measurements without cellular disruption (Knight et al., 1991). This technology is the most suitable and popular tool to measure Ca^{2+} changes in whole intact plants. Targeting of the reconstituted aequorin photoprotein to distinct subcellular locations allows not only monitoring changes in Ca^{2+} concentrations in different organelles but also at cellular subcompartments such as regions adjacent to the membranes, in regions referred to as *microdomains* or other specific cellular sites (Rudd and Franklin-Tong, 2001). Aequorin has been successfully targeted to the chloroplast (Johnson et al., 1995), nucleus (Badminton et al., 1998), endoplasmic reticulum (Kendall et al., 1994), vacuoles (Knight et al., 1996; Allen et al., 1995), and mitochondria (Montero et al., 2000) using targeting sequences consisting of either peptide leader sequences or whole polypeptides encoding proteins that exists naturally in the specific microdomain. The intensive use of recombinant aequorin technology in plants is due to its versatility, its high dynamic range of measurement from 10 nM up to 10 μM, no buffering capacity or toxicity, very low leakage rate from cells, absence of intracellular compartmentalization of the natural aequorin protein, and the potential for accurate and quantitative measurements. However, the $(Ca^{2+})_{cyt}$ changes are the average of many cells and do not necessarily reflect the (Ca^{2+}) fluctuations in individual cells. The emitted light is expressed as relative luminescence units (RLUs) using a luminometer. In each measurement, the concentration of reconstituted aequorin should not be limiting and not exceed 10% of the maximal possible Ca^{2+}-binding capacity. The remaining reconstituted aequorin is estimated by a discharge reaction by adding excess $CaCl_2$ in

10% ethanol (v/v) (e.g., 100 mM for roots or 1–2 M for leaves of *Arabidopsis* seedlings (Johnson et al., 2011a). The luminescence measured during this discharge is used to calculate the total amount of aequorin present in the cells/tissues (Fig. 79.1). The discharge measurement also ensures that the amount of aequorin is not limiting for the specific Ca^{2+} signal measured after the stimulus. The RLUs are converted into Ca^{2+} concentrations by a calibration curve (Moyen et al., 1998).

79.5 CA^{2+} SIGNALING IN PLANT–FUNGUS INTERACTIONS

Ca^{2+} signals are one of the earliest responses in root and leaf cells in response to pathogenic and symbiotic fungi. These signals are key determinants for the development of symbiosis, disease resistance, or susceptibility in plants (Chabaud et al., 2011; Vidhyasekaran, 2008; Garcia-Brügger et al., 2006). Ca^{2+} elevation in plant cells begins within seconds after the contact with fungi and sometime even before physical contact of the two symbionts. This suggests that the Ca^{2+} response in the plant cells is elicited by exudate components released by the microbes. The main question is how the plant cell establishes the appropriate Ca^{2+} response to pathogenic or beneficial fungi. The outcome of the plant–fungus interaction (e.g., beneficial symbiosis, susceptibility or resistance) may be determined during the very first few minutes of association (Heath, 2000), for example, by activating a receptor, which establishes a specific, response-determining Ca^{2+} signature in the cell. Alternatively, the mode of the symbiosis might be the result of a longer coexistence in which the two symbionts decide gradually on the mode of interaction (Harrison, 2005). This may include also that a fungus releases more than one signal that triggers $(Ca^{2+})_{cyt}$ elevation in the plant cell, or a certain degree of unspecificity in the plant response to the fungus-induced elevated $(Ca^{2+})_{cyt}$.

Both pathogen-associated molecular patterns (PAMPs) and beneficial microbe-associated molecular patterns (bMAMPs) transiently increase $(Ca^{2+})_{cyt}$ in their host cells within seconds after the activation of the respective receptor(s). Specificity in signaling might be achieved by differences in the $(Ca^{2+})_{cyt}$ signatures induced by PAMP and bMAMPs, for example, by differences in its strength, space, time or spiking. Furthermore, PAMPs and bMAMPs could mobilize Ca^{2+} from different stores. It is also known that defense responses are both suppressed and activated by Ca^{2+} signals suggesting the presence of Ca^{2+}-responsive and antagonistic signaling mechanisms (Dodd et al., 2010). (cf. Table 79.2).

79.5.1 Ca^{2+} Signaling in Fungal Cells: Plant–Pathogenic Fungal Interactions

Changes in $(Ca^{2+})_{cyt}$ in plant cells in response to pathogens and their PAMPs have been widely reported (Ranf et al., 2011; Lecourieux et al., 2006; Klüsener et al., 2002; Blume et al., 2000; Zimmermann et al., 1997; Dixon et al., 1994). Cytosolic Ca^{2+} is also important for hyphal tip growth and appressorial development in many fungi (Jackson and Heath, 1993). The involvement of Ca^{2+} signaling in the appressorium formation has been reported for *Colletotrichum gloeosporioides* (Kim et al., 1998). Several inhibitors interfering with the Ca^{2+}-signaling system have been shown to inhibit appressorium formation of *C. gloeosporioides* (Uhm et al., 2003). TMB-8 (3,4,5-trimethoxybenzoic acid 8-(diethyl-amino)octyl ester), an inhibitor of Ca^{2+} release from intracellular Ca^{2+} stores such as organelles, and methoxy verampamil, a Ca^{2+} channel blocker inhibited appressorium formation in *C. gloeosporioides* (Uhm et al., 2003). These results

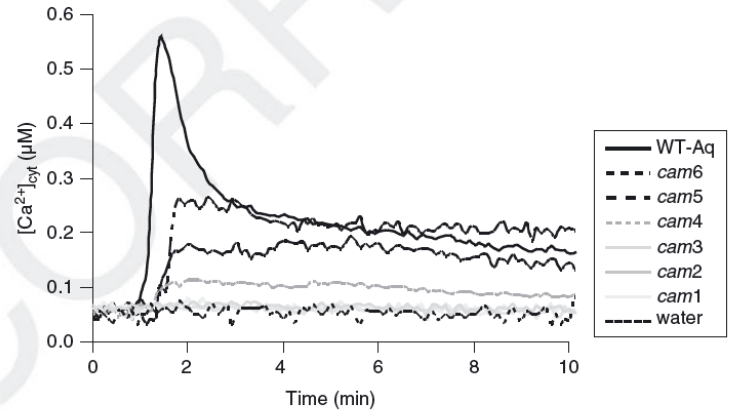


Figure 79.1 Mutants less or not at all responding to *Piriformospora indica* CWE-induced $(Ca^{2+})_{cyt}$ elevation. Transgenic Arabidopsis roots with reconstituted aequorin have a basal $(Ca^{2+})_{cyt}$ level between 0.04 to 0.06 µM. When the CWE was added, after initial lag phase of 30–40 s, a rapid and transient elevation of the $(Ca^{2+})_{cyt}$ up to 0.6 µM was observed between 1 and 2 min followed by steady and sustained decrease. The concentration declined gradually and reached its basal level 80–90 min after CWE application. The mutant *cam1, cam2 and cam3* did not respond at all to the *P. indica*-CWE whereas *cam4, cam5 and cam6* showed 50–90% reduction in $(Ca^{2+})_{cyt}$ elevation. Sterile water served as control.

Table 79.2 Different physiological responses and processes initiated by changes in $(Ca^{2+})_{cyt}$ due to various PAMPs/MAMPS

PAMPs/MAMPs	Origin	$(Ca^{2+})_{cyt}$ response	Cellular/ physiological process	Selected references
Cryptogein (Elicitins)	*Phytophthora cryptogea*	Transient $(Ca^{2+})_{cyt}$ rise in 6 min & later small increase at 20 min	MAPK, H_2O_2, NO, HR, defense	Lecourieux et al. (2002), Lecourieux et al. (2006)
β-1,3-Glucan	*Phytophthora sojae*	Transient $(Ca^{2+})_{cyt}$ rise	Phytoalexin defense	Mithöfer et al. (1999)
Pep-13	*Phytophthora sojae*	Transient $(Ca^{2+})_{cyt}$ rise after 2 min	Phytoalexin, ROS, defense	Blume et al. (2000)
Pep-25	*Phytophthora sojae*	Transient $(Ca^{2+})_{cyt}$ rise	H_2O_2, defense	Hu et al. (2009)
INF1	*Phytophthora infestans*	Transient $(Ca^{2+})_{cyt}$ elevation	Stomatal regulation, ROS, defense	Zhang et al. (2009)
Boehmerin	*Phytophthora boehmeriae*	Transient $(Ca^{2+})_{cyt}$ elevation	Stomatal regulation, ROS, defense	Zhang et al. (2009)
Endopolygalacturonase (BcPG1)	*Botrytis cinerea*	Biphasic and sustained $(Ca^{2+})_{cyt}$ elevation	NO, defense	Vandelle et al. (2006)
Yeast elicitor	*Saccharomyces cerevisiae*	Repetitive $(Ca^{2+})_{cyt}$ transients	Stomatal regulation, ROS	Klüsener et al. (2002)
Avr5	Cladopsorium fulvum	Transient $(Ca^{2+})_{cyt}$ elevation	Defense	Gelli et al. (1997)
Laminarin (β-1,3-glucan)	*Laminaria digitata*	Transient $(Ca^{2+})_{cyt}$ rise in 1–2 min & slow increase in 4 min	MAPK, defense	Lecourieux et al. (2002), Lecourieux et al. (2006)
Chitooligosaccharide	*Pseudomonas* spp.	Transient $(Ca^{2+})_{cyt}$ rise in 1–2 min & later increase in 4-5 min	HR, defense	Müller et al. (2000), Lecourieux et al. (2006)
Lipopolysaccharide	*Pseudomonas* spp.	Transient $(Ca^{2+})_{cyt}$ rise in 1–2 min & later increase in 4 min	HR, defense	Lecourieux et al. (2002), Lecourieux et al. (2006)
Flagellin 22	Flagellated bacteria	Transient $(Ca^{2+})_{cyt}$ rise for 1–7 min	Defense	Jeworutzki et al. (2010)
avrRpm1	*Pesudomonas syringae pv. tomato*	Transient $(Ca^{2+})_{cyt}$ rise for 10 min & second transient increase for 2h	Hypersensitive cell death	Grant et al. (2000), Lecourieux et al. (2002)
Lipopolysaccharide	*Burkholdaria cepacia*	Transient $(Ca^{2+})_{cyt}$ rise	Oxidative burst, NO, defense	Gerber et al. (2004)
Harpin P_{sph}	*Pesudomonas syringae pv. phaseolina*	Transient $(Ca^{2+})_{cyt}$ rise	HR, defense	Lee et al. (2001)
C4-HSL	Gram negative bacteria	Sudden transient $(Ca^{2+})_{cyt}$ rise	Quorum sensing perception	Song et al. (2011)
Nod factor (Lipochitin oligomers)	Rhizobia	Initial $(Ca^{2+})_{cyt}$ rise and then oscillations of $(Ca^{2+})_{cyt}$	Nodulation and symbiosis, Root hair curling	Ehrhardt et al. (1996), Müller et al. (2000)
Myc factor (Lipochi-tooligosaccharides)	AM fungi (*Glomus* spp.)	Transient, then slow sustained $(Ca^{2+})_{cyt}$ elevation and oscillations	Mycorrhiza formation, root hair curling and symbiosis	Navazio et al. (2007), Kosuta et al. (2008)
Pi-CWE	*Piriformospora indica*	Transient, then slow sustained $(Ca^{2+})_{cyt}$ elevation and oscillations	Growth promotion and symbiosis	Vadassery et al. (2009)

suggest that Ca^{2+} channels are involved in the signal transduction pathway to induce germination and appressorium formation in fungal pathogens (Uhm et al., 2003). Fungal calmodulin is also involved in appressorium formation in *C. gloeosporioides* (Kim et al., 1998) and *C. trifolii* (Buhr and Dickman, 1997). Chloropromazine, a calmodulin antagonist, which competes with Ca^{2+} for binding to calmodulin, inhibits appressorium formation in *C. gloeosporioides* (Uhm et al., 2003). Addepalli and Fujita (2002) found that Ca^{2+} is absolutely required for the zoosporangia formation, release of zoospores, and their germination in an oomycete, *Pythium porphyrae*. The Ca^{2+} release from internal Ca^{2+} stores appears to be more important than plasma membrane Ca^{2+} influx for the appressorium formation in *Magnaporthea grisea* (Lee and Lee, 1998) and *C. gloeosporioides* (Uhm et al., 2003). Further studies revealed that proteins involved in Ca^{2+} signaling including phospholipase C are also required for appressorium formation, host penetration, establishment of mycelial growth in the host cell, and conidial formation in *M. oryzae* (Choi et al., 2011; Choi et al., 2009a; 2009b). RNA interference studies clearly indicate that Ca^{2+} transporters, viz. ion channels, pumps, and exchangers are involved in asexual reproduction and infection processes in *M. oryzae* (Nguyen et al., 2008).

79.5.1.1 Ca^{2+} Signaling in Plant Defense.

Changes in $(Ca^{2+})_{cyt}$ homeostasis are an essential early step in pathogen perception and subsequent innate immune response of plant cells (Ma and Berkowitz, 2007; Dangl et al., 1996). Elicitation of defense responses by pathogens and their PAMPs is triggered by $(Ca^{2+})_{cyt}$ elevation (Ebel et al., 1995). PAMP treatment increases $(Ca^{2+})_{cyt}$ and it plays a pivotal role in activating the plant's surveillance system against attempted pathogen invasion (Table 79.2) (Klüsener et al., 2002; Nürnberger and Scheel, 2001). Transient changes in permeability of the plasma membrane to Ca^{2+} are a common early event in plant defense signaling (Wendehenne et al., 2002; Jabs et al., 1997; Atkinson et al., 1996). Therefore, elicitor-responsive Ca^{2+}-permeable ion channels located at the plasma membrane of plant cells mediate elicitor-induced Ca^{2+} entry, resulting in transient changes in $(Ca^{2+})_{cyt}$ (Lecourieux et al., 2006; 2002). Chitosan and yeast elicitor activate a hyperpolarization-dependent current in *Arabidopsis* cells (Klüsener et al., 2002). A race-specific elicitor (Avr5) from *Cladosporium fulvum* induces the activation of a Ca^{2+}-permeable channel in the plasma membrane (Gelli et al., 1997). Massive influx of Ca^{2+} in tobacco-cultured cells was observed within 15–30 min after treatment with the cryptogein elicitor (Lecourieux-Ouaked et al., 2000). Recognition and perception of PAMPs through specific receptors by plant cells induces $(Ca^{2+})_{cyt}$ elevation, which leads to modulation of the defense-signaling cascades (Fig. 79.2). High affinity binding of PAMPs viz. cryptogein and Pep-13 to plasma membrane receptor proteins of tobacco and parsley cells have been reported (Bourque et al., 1999, 1998, Nürnberger et al., 1994). It has been demonstrated that the Pep-13-stimulated increase in $(Ca^{2+})_{cyt}$ is a receptor-mediated process using a series of structural derivatives of Pep-13 (Blume et al., 2000).

The rapid production of reactive oxygen species (ROS) and nitric oxide (NO) is an early inducible plant

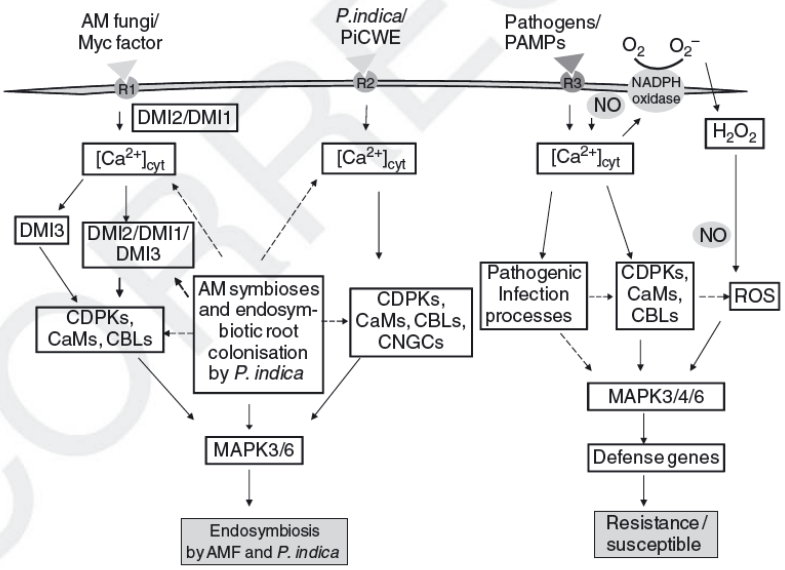


Figure 79.2 General scheme of events in beneficial and pathogenic plant–fungus interactions. Fungi and their PAMPs or bMAMPs are perceived by plasma membrane-associated receptors leading to transient $(Ca^{2+})_{cyt}$ elevation. In beneficial interactions, $(Ca^{2+})_{cyt}$ elevation leads to activation of *SYM* genes viz. *DMI1/2/3* in AM symbiosis. Various Ca^{2+} sensors, decoders and transducers-like CDPKs, CaMs, CBLs, CNGCs and MAPKs are involved in establishing endosymbiosis by AMF or *P. indica*. In pathogenic interactions, $(Ca^{2+})_{cyt}$ elevation leads to the activation or suppression of NADPH-oxidase, ROS formation, different Ca^{2+} sensors, decoders and transducers. This results in the activation or suppression of defense genes involved in disease resistance or susceptibility.

defense response during pathogen invasion or on treatment with PAMPs (Table 79.2) (Zhang et al., 2009; Wendehenne et al., 2004; Klessig et al., 2000; Wojtaszek, 1997).

Both Ca^{2+} fluxes and protein phosphorylation are required for the controlled generation of H_2O_2 (Neill et al., 2002). ROS signal transduction activates Ca^{2+} channels (Mori and Schroeder, 2004). PAMPs induce both $(Ca^{2+})_{cyt}$ increase and ROS generation (Fig. 79.2). In some cases, $(Ca^{2+})_{cyt}$ elevations have been reported upstream of ROS production (Kawano and Muto, 2000); in other cases, Ca^{2+} elevations occur downstream of ROS production (Blume et al., 2000; Bowler and Fluhr, 2000), indicating complex spatiotemporal Ca^{2+} elevation mechanisms. Membrane-bound NADPH oxidases are involved in ROS production (Mori and Schroeder, 2004) and PAMP-induced elevation in $(Ca^{2+})_{cyt}$ lies upstream of NADPH-oxidase activation (Blume et al., 2000). The relationship between the NADPH oxidase-dependent H_2O_2 production and a subsequent $(Ca^{2+})_{cyt}$ elevation was reported in the cryptogein/tobacco system (Lecourieux et al., 2002), and demonstrated through stimulation of an NADPH-dependent, hyperpolarization-activated Ca^{2+} influx in *Arabidopsis* guard cells treated with yeast elicitors or chitosan (Klüsener et al., 2002).

Interestingly, the AtRbohD and AtRbohF (for *Arabidopsis* respiratory burst oxidase homolog)-NADPH oxidases have a dual function of mediating pathogen-associated ROS production and ABA signal transduction (Kwak et al., 2003; Torres et al., 2002). Furthermore, Rboh proteins have hydrophilic N-terminal regions containing two EF-hand motifs, suggesting that their activation is dependent on Ca^{2+}. Ogasawara et al. (2008) have shown that ROS production by *Arabidopsis thaliana* RbohD was induced by ionomycin, which is a Ca^{2+} ionophore that induces Ca^{2+} influx into the cell. This activation required a conformational change in the EF-hand region, as a result of Ca^{2+} binding to the EF-hand motifs. AtrbohD was also directly phosphorylated *in vivo*. Conclusively, Ca^{2+} binding and phosphorylation synergistically activate the ROS-producing enzyme activity of AtrbohD.

Cryptogein treatment in tobacco cells induced a very fast Ca^{2+}-dependent NO production (Lamotte et al., 2004; Foissner et al., 2000). Characterization of AtNOS1 (*A. thaliana* NO synthase) has shown that the enzyme contains calmodulin (CaM)-binding motifs and the full activation of AtNOS1 requires both Ca^{2+} and CaM (Guo et al., 2003). It is postulated that NO promotes $(Ca^{2+})_{cyt}$ elevations through the mobilization of intracellular pools of Ca^2 (Vandelle et al., 2006; Wendehenne et al., 2004).

A better understanding of the physiological significance of the $(Ca^{2+})_{cyt}$ changes in different plant–pathogen interactions involves the identification and functional analysis of downstream targets of $(Ca^{2+})_{cyt}$, which includes the regulation of protein kinases, protein phosphatases, phospholipases, G-proteins, NADPH oxidases, ion channels, and the production of various metabolites such as ROS, NO, salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) to activate defense gene expression (Dangl and Jones, 2001; Nürnberger and Scheel, 2001; Lamb et al., 1989). Posttranslational modification of proteins by reversible phosphorylation is a key process regulating defense responses induced by PAMPs (Peck et al., 2001; Lecourieux-Ouaked et al., 2000; Dietrich et al., 1994; 1990; Felix et al., 1991). Phosphorylation events have been described both upstream and downstream of PAMP-induced Ca^{2+} influxes (Fig. 79.2). For example, defense-related responses including the early Ca^{2+} influx are prevented by the general serine/threonine protein kinase inhibitor staurosporine in cryptogein-treated tobacco cells (Lecourieux et al., 2002). The authors also showed that the cryptogein-induced $(Ca^{2+})_{cyt}$ increase is fully inhibited by staurosporine. MAPK cascades that transduce extracellular stimuli into intracellular responses were shown to be associated with the induction of plant defense responses (Jonak et al., 2002; Zhang and Klessig, 2001). Pharmacological studies indicated that the Ca^{2+} signaling is upstream of MAPK pathways in plant defence responses (Link et al., 2002; Romeis et al., 1999). Normally, both $(Ca^{2+})_{cyt}$ elevations and MAPK activation do not exceed 15 min whereas cryptogein treatment leads to a fast and sustained MAPK activation for at least 2 h, which is in accordance with the cryptogein-induced $(Ca^{2+})_{cyt}$ increase in aequorin-transformed tobacco cells (Table 79.2) (Lecourieux et al., 2002). Further, suppression of the sustained $(Ca^{2+})_{cyt}$ increase in cryptogein-treated cells abolished the activation of MAPKs (Lecourieux et al., 2002), and the overexpression of a putative voltage-gated Ca^{2+} channel in rice resulted in enhanced activation of an MAPK cascade on PAMP treatment (Kurusu et al., 2005).

It is well established that $(Ca^{2+})_{cyt}$ elevation alters defense gene expression, phytoalexin accumulation, and HR-related cell death (Fig. 79.2). Changes in defense gene expression such as for pathogenesis-related (PR) proteins with antimicrobial activities and for different enzymes involved in the phenylpropanoid biosynthetic pathways leading to the production of phenolics, polyphenolics, lignins, and phytoalexins have been reported in numerous plant–pathogen interaction systems due to $(Ca^{2+})_{cyt}$ elevation (Ebel, 1995; Tavernier et al., 1995; Stäb and Ebel, 1987). Using aequorin-expressing cells, it has been demonstrated that the PAMP-induced increase in $(Ca^{2+})_{cyt}$ is involved in accumulation of phytoalexins. A prolonged $(Ca^{2+})_{cyt}$ elevation appears to be correlated with phytoalexin accumulation in soybean or parsley cells (Blume et al., 2000; Mithöfer et al., 1999). Interestingly, suppression of the sustained $(Ca^{2+})_{cyt}$ increase in

cryptogein-treated tobacco cells suppressed the accumulation of phenylalanine ammonia lyase (*PAL*) transcripts. The enzyme mediates the first specific biosynthetic step in the phenylpropanoid pathway (Lecourieux et al., 2002). A slow and prolonged elevation in $(Ca^{2+})_{cyt}$ is involved in establishment of the HR in cowpea and tobacco (Lecourieux et al., 2002; Sasabe et al., 2000; Xu and Heath, 1998). An extended elevation of $(Ca^{2+})_{cyt}$ in resistant but not susceptible cowpea plants after inoculation with basidiospores of the rust fungus *Uromyces vignae* resulted in a hypersensitive reaction (Xu and Heath, 1998).

A large number of Ca^{2+} sensors including CDPKs, CaM, CBL, and Ca^{2+}-regulated protein kinases, which decode Ca^{2+} signatures are also involved in plant defense against fungal pathogens and their PAMPs (Fig. 79.2) (Lamotte et al., 2004; Cheng et al., 2002; Lee and Rudd, 2002; Yang and Poovaiah, 2002). Incision of mildew resistance locus o (MLO), a transmembrane CaM-binding protein in barley, leads to powdery mildew resistance and to a deregulated leaf cell death, indicating that MLO has a negative regulatory function in plant defense and cell death (Kim et al., 2002). A rapid $(Ca^{2+})_{cyt}$ elevation in barley epidermal cells on mildew infection reinforced the MLO-dependent defence suppression via a Ca^{2+}/CaM-dependent process. Nevertheless, the powdery mildew-resistant *mlo* plants exhibited enhanced susceptibility to *Magnaporthe grisea* and *Bipolaris sorokiniana* (Kumar et al., 2001; Jarosch et al., 1999). A possible explanation is that *mlo* mutations modulate different resistance mechanisms effective against biotrophic or necrotrophic fungi (Panstruga and Schulze-Lefert, 2003). Several *CDPK* transcripts accumulate in tobacco, maize, tomato, or pepper in response to fungal infection and treatment with PAMPs (Chung et al., 2004; Chico et al., 2002; Romeis et al., 2000).

79.5.2 Ca^{2+} Signaling in the Interaction of Roots with Beneficial Microbes

Arbuscular mycorrhizal (AM) fungi and *Piriformospora indica*, an endophytic root-colonizing nonAM fungus, form mutually beneficial symbiotic association with many plants (cf. Smith and Smith, 2011; Oelmüller et al., 2009). AM fungi colonize nearly 80% of the terrestrial plant species and are obligately symbiotic fungi in the phylum Glomeromycota, whereas the endophytic nonobligately symbiotic *P. indica* colonizes almost all plant species tested so far. *P. indica* belongs to the order Sebacinales in the phylum Basidiomycota (cf. Smith and Smith, 2011; Oelmüller et al., 2009). Both fungal symbionts provide nutrients like nitrogen, phosphorus, and micronutrients to host plants in exchange for photosynthates and a privileged ecological niche essential for fungal development and propagation (Smith and Smith, 2011; Yadav et al., 2010; Smith and Read, 2008; Sherameti et al., 2005). AM fungi form a varied range of structures in the colonized roots like appressorium, extra- and intraradical mycelia, intercellular hyphae, dichotomously branched intracellular arbuscules, and intracellular hyphal tips (Pumplin and Harrison, 2009; Genre et al., 2008; Karandashov et al., 2004; Smith and Smith, 1997), whereas *P. indica* forms extra-, inter-, and intracellular hyphae and chlamydospores in the colonized roots (Johnson et al., 2011b; Vahabi et al., 2011; Zuccaro et al., 2011; Deshmukh et al., 2006; Waller et al., 2005; Varma et al., 1999; 2001).

As both types of symbiotic fungi can colonize the roots of many plant species, the interaction should be based on general recognition and signaling processes. The root must recognize the interacting fungi as symbionts and not as pathogens. The complex cellular interaction between root and fungi necessitates continuous recognition and signal exchange between both partners (Bonfante and Requena, 2011; Camehl et al., 2011; Smith and Smith, 2011; Vadassery et al., 2009; Bonfante and Genre, 2008; Johnson and Oelmüller, 2009; Parniske, 2008; Paszkowski, 2006; Harrison, 2005). The signaling starts prior to the physical contact between the partners; both plant and fungus perceive diffusible molecules released by the counterpart as signals to initiate symbiotic association (Bécard et al., 2004). As a consequence of the signal perception, activation of specific genes and morphogenetic programs occur both in the plant (Vadassery et al., 2009; Kosuta et al., 2008; 2003; Parniske, 2008; Genre et al., 2005; Harrison, 2005; Oláh et al., 2005; Weidmann et al., 2004) and in the fungus (Chabaud et al., 2011; Zuccaro et al., 2011; Deshmukh et al., 2006; Waller et al., 2005; Breuninger and Requena, 2004; Tamasloukht et al., 2003). An early event in this recognition and initiation of symbiosis is the triggering of $(Ca^{2+})_{cyt}$ elevation (Fig. 79.2). Both fungal symbionts and their bMAMPs induce $(Ca^{2+})_{cyt}$ elevation in host plants during symbiosis (Table 79.2) (Johnson et al., 2011a; Vadassery et al., 2009; Kosuta et al., 2008; Navazio et al., 2007). The interaction of AM and beneficial fungi with roots results in better plant performance through sequential cytoplasmic and nuclear Ca^{2+} ($(Ca^{2+})_{nuc}$) elevations (Vadassery et al., 2009; Kosuta et al., 2008; Navazio et al., 2007).

Interestingly, in AM and nodule formation, the Ca^{2+} signaling is extended to the nucleus. Rhizobacteria and AM fungi induce a nucleus associated Ca^{2+} spiking response, which is decoded by the plant-specific Ca^{2+}- and calmodulin-dependent kinase CCaMK (Groth et al., 2010; Kosuta et al., 2008; Oldroyd and Downie, 2008).

ccamk mutants initiate Ca^{2+} spiking in response to the symbiotic microbes, but expression of early symbiosis genes and the establishment of nodules and AM symbiosis is impaired. CCaMK contains a kinase domain linked to a calmodulin-binding site and three Ca^{2+}-binding EF hands. The activation of CCaMK depends on either free Ca^{2+} or activated calmodulin, whereas differences in the Ca^{2+} signatures participate in decoding the Ca^{2+} information to AM fungi symbiosis or nodule formation (Gleason et al., 2006; Tirichine et al., 2006). A point mutation in the autophosphorylation site of CCaMK results in nodule formation even in the absence of rhizobia. Thus, CCaMK functions as a regulatory switch for nodule formation (Groth et al., 2010; Gleason et al., 2006; Tirichine et al., 2006). CCaMK assembles with CYCLOPS in the nucleus prior to Ca^{2+} binding (Yano et al., 2008) and *cyclops* mutants are defective in mycorrhiza and nodule formation, but their Ca^{2+} spiking response was not impaired (Yano et al., 2008; Miwa et al., 2006; Kistner et al., 2005).

79.5.2.1 Cytoplasmic Ca^{2+} Signaling in AM Symbiosis. AM fungi establish mutualistic interactions with roots of most terrestrial plants and play a major role in nutrient uptake and nutrient cycling of their hosts, influence growth, water absorption, and protection against root diseases (Smith and Read 2008). $(Ca^{2+})_{cyt}$ transients occur in root cells during the formation of the symbiotic relationships (Chabaud et al., 2011; Kosuta et al., 2008; Navazio et al., 2007). Rapid and transient elevations of $(Ca^{2+})_{cyt}$ during the early phase of AM symbiosis indicate that AM diffusible molecules constitutively released by the mycorrhizal fungus are perceived by the host cells (Navazio et al., 2007). The Ca^{2+} signal induced by these diffusible molecules is decoded into a biphasic Ca^{2+} trace characterized by a rapid high elevation in $(Ca^{2+})_{cyt}$, which is followed by a smaller transient increase that is completely dissipated within 30 min (Navazio et al., 2007). Upregulation of *Medicago truncatula DMI1*, *DMI2*, and *DMI3* genes essential for the establishment of the AM symbiosis in soybean cells fails to induce $(Ca^{2+})_{cyt}$ elevation in cultured cells of the nonhost plant *Arabidopsis* suggesting specificity of the fungal signals to plants that form AM symbiosis (Navazio et al., 2007; Breuninger and Requena, 2004). Diffusible molecules released by germinating spores evoke a transient $(Ca^{2+})_{cyt}$ elevation without triggering defense responses (Fig. 79.2). This could be a general trait of AM fungi to initiate the symbiotic association with different terrestrial plants (Navazio et al., 2007).

Soon after spore germination, AM fungi produce intense hyphal ramification to contact the host roots and further differentiate into fungal adhesion structures, known as *hyphopodia*, on nonroot hair epidermal cells called *atrichoblasts* (Parniske, 2008; Harrison, 2005). In hyphopodia-contacted host root cells, the organization of a polarized cytoplasmic assembly termed the *prepenetration apparatus* (PPA) is formed. Initiation of PPA formation requires $(Ca^{2+})_{cyt}$ signaling (Parniske, 2008; Genre et al., 2005). PPA is inevitable for the AM formation and successful symbiosis as the *dmi* mutants of *Medicago truncatula* defective in both PPA formation and infection failed to form AM structures (Parniske, 2008; Genre et al., 2005). Chabaud et al. (2011) demonstrated Ca^{2+} signaling responses in hyphopodia-contacted atrichoblasts of both *M. truncatula* and carrot roots before infection. This suggests that Ca^{2+} spiking is a key component of a highly conserved AM-activated signaling pathway, which is required for intracellular fungal infection/colonization.

Ca^{2+} signaling also plays a pivotal role during the early stages of the AM association. Symbiotic AM diffusible signals (Myc factors) induce common *SYM* genes and DMI1/2-dependent cytoplasmic Ca^{2+} spiking in root hairs of *M. truncatula* (Fig. 79.2) (Horvàth et al., 2011; Kuhn et al., 2010; Gutjahr et al., 2009; Kosuta et al., 2008; Navazio et al., 2007). This is independent of the *NFP* gene encoding the lysine motif-receptor-like kinase (LysM RLK), which mediates nod factor (NF) and DMI3 perception (Arrighi et al., 2006; Lévy et al., 2004). Ca^{2+} spiking induced by the AM fungal exudate exhibits shorter and smaller amplitudes compared to the NF-induced response (Kosuta et al., 2008; 2003). Diffusible AM signals are involved in a variety of host responses including the activation of *SYMBIOSIS* (*SYM*) genes (Kuhn et al., 2010; Kosuta et al., 2003), the stimulation of lateral root branching (Olah et al., 2005), and the activation of starch-related metabolic pathways (Gutjahr et al., 2009) in *M. truncula*. Many of these responses appear to be dependent on the common symbiosis (SYM) pathway (Kuhn et al., 2010; Gutjahr et al., 2009; Olah et al., 2005). The diffusible molecules of the AM fungi named Myc factors assist the plant in recognizing the fungi as friends and not as foes (Bonfante and Requena, 2011). They were identified recently as a mixture of sulphated and nonsulfated simple lipochitooligosaccharides (LCOs), which stimulate formation of AM in different plant species (Maillet et al., 2011). Further, the authors showed that these signals stimulate root growth and branching by the symbiotic DMI signaling pathway, and that they slightly induce the transcription of some AM-responsive genes in legume and nonlegume plants (Maillet et al., 2011). Therefore, plant perception of AM fungal diffusible signals is translated into transcriptional responses that initiate and prepare the plant for fungal accommodation (Bonfante and Requena, 2011). AM fungal diffusible factors do not induce any defense responses like ROS accumulation and cell death in host cells (Navazio et al., 2007).

79.5.2.2 Ca^{2+} Signaling in* Arabidopsis *and* P. indica *Symbiosis. The beneficial interaction of *P. indica* with a multitude of horticulturally and agriculturally important plants as well as model plants including *Arabidopsis thaliana* ultimately leads to growth promotion, increased biomass production, and enhanced resistance/tolerance against biotic and abiotic stress (Johnson et al., 2011b; Lee et al., 2011; Sun et al., 2010; Shahollari et al., 2007; Sherameti et al., 2005; Waller et al., 2005; Varma et al., 2001; 1999). The fungus can be cultivated axenically on synthetic or complex media without a host (Johnson et al., 2011b; Varma et al., 1999; 2001). Once inside the roots, the fungus gets access to photoassimilates and other plant nutrients, which further promotes colonization and proliferation. Moreover, the fungus significantly enhances root growth thus providing more niches for growth (Oelmüller et al., 2009). The plants benefit from this relationship by reprogramming plant transcriptomes, proteomes, and metabolomes that are directly or indirectly involved in phytohormone synthesis and signaling, increased root and shoot growth, increased nutrient uptake, early flowering, enhanced seed production, and protection against drought, salinity, root and foliar pathogens (Camehl et al., 2011; 2010; Zuccaro et al., 2011; Yadav et al., 2010; Vadassery et al., 2009; 2008; Sherameti et al., 2008; 2005; Stein et al., 2008; Shahollari et al., 2007; Deshmukh et al., 2006; Waller et al., 2005). The modulation of gene expression, of proteins and metabolites, helps both partners to keep the interaction mutually beneficial. The endophytic interaction of *P. indica* with the model plant *A. thaliana* helps to understand the molecular and physiological basis of such symbioses. To study this interaction, both partners should be grown together in harmony (Johnson et al., 2011b).

As the fungus can colonize the roots of all plant species tested so far, the interaction between the symbiotic partners should be based on general recognition and signaling processes (Oelmüller et al., 2009). Ca^{2+} plays a major role in the interaction between *P. indica* and *A. thaliana*. One of the earliest signaling events is a transient increase of $(Ca^{2+})_{cyt}$ followed by a nuclear Ca^{2+} response (Vadassery et al., 2009). A heat stable cell wall extract (CWE) from the fungus promotes the growth of wild-type seedlings but not of seedlings of *P. indica*-insensitive mutants (Fig. 79.1). The *P. indica* inducible marker genes are not upregulated in mutants either colonized with the fungus or treated with the CWE. The extract and the fungus induce similar sets of genes in *Arabidopsis* roots, which also include genes with Ca^{2+} signaling-related functions (Vadassery et al., 2009). Therefore, it is assumed that growth promotion and $(Ca^{2+})_{cyt}$ elevation must be linked (Table 79.2). The CWE induces $(Ca^{2+})_{cyt}$ elevation preferentially in the roots and marginally in the shoots, which is consistent with the fact that the endophyte is a root-colonizing fungus (Vadassery and Oelmüller, 2009).

Ca^{2+} signaling in plant–microbe interaction is often distributed between different cellular subcompartments and/or organelles (Pauly et al., 2001). In rhizobial and AM symbiosis, both $(Ca^{2+})_{cyt}$ and $(Ca^{2+})_{nuc}$ elevations are crucial for the establishment of symbiotic relationships. The CWE from *P. indica* also induce distinct $(Ca^{2+})_{cyt}$ and $(Ca^{2+})_{nuc}$ elevations in BY-2 tobacco cell cultures expressing apo-aequorin either in the cytoplasm or in the nucleus (Vadassery et al., 2009). The maximum $(Ca^{2+})_{cyt}$ elevation occurs within 2 min, whereas $(Ca^{2+})_{nuc}$ elevation is detected after 6 min, suggesting a sequential response (Vadassery et al., 2009). Different studies with Ca^{2+} signaling inhibitors and the refractory behavior of the response to the CWE suggested that the fungal stimulus is perceived by a plant receptor (Vadassery and Oelmüller, 2009). Cells do not respond to subsequent treatments with the same stimulus within a certain time frame ("refractory behavior"), but remain sensitive to a different stimulus (Müller et al., 2000). Moreover the transcript levels for a leucine-rich repeat protein LRR1 and the glutamate receptor GLR2.5, two important proteins involved in *P. indica* interaction, are strongly upregulated in roots of colonized and CWE-treated roots (Shahollari et al., 2007; Vadassery et al., 2009).

Protein phosphorylations are key processes regulating defense downstream elicitor-induced Ca^{2+} influx (Dietrich et al., 1990). Both Mitogen-Activated Protein Kinase (MAPK)3 and MAPK6 are activated by the fungus and its CWE, and this response is more pronounced in roots than in shoots (Vadassery et al., 2009). Ca^{2+} inhibitors such as $LaCl_3$ and BAPTA inhibit $(Ca^{2+})_{cyt}$ elevation and also abolish MAPK phosphorylation. Furthermore, the *mpk6* knockout line does not respond to *P. indica* suggesting an involvement of this kinase in the beneficial interaction (Fig. 79.2) (Vadassery et al., 2009).

The CNGCs maintain cation homeostasis in plant cells. Both the fungus and its CWE upregulate *CNGC10*, which codes for protein localizes to the plasma membrane. It influences growth responses and starch accumulation (Borsics et al., 2007; Christopher et al., 2007). Similar regulation was observed for the closely related *CNGC13* gene (Vadassery et al., 2009). Downregulation of *CNGC2/DND1* involved in the generation of NO and the innate immune response to pathogens (Ali et al., 2007) and of CNGC11, for a regulator of resistance against fungal pathogens (Yoshioka et al., 2006), supports the idea that the fungus and its CWE suppress defense gene activation (Vadassery et al., 2009). Genes for different Ca^{2+} sensors like the calmodulin-like proteins CML42, CML38, and CML30 and for

CBP20, a calmodulin-binding protein involved in disease resistance is upregulated by both stimuli in Arabidopsis (Vadassery et al., 2009). The upregulation of Ca^{2+} signaling genes supports the importance of Ca^{2+} in this interaction (Fig. 79.2). On the other hand, the CWE did not induce H_2O_2 production, an oxidative burst, programmed cell death and hypersensitive reactions in Arabidopsis root (Sherameti et al., 2008; Vadassery et al., 2009).

79.6 CONCLUSIONS

Ca^{2+} signaling plays an important role in the initial recognition processes and also in the subsequent chain of events leading to either symbiosis or disease development. Both beneficial and pathogenic fungi, and their bMAMPs and PAMPs induce distinct Ca^{2+} signatures in terms of $(Ca^{2+})_{cyt}$ and $(Ca^{2+})_{nuc}$ elevations through specific receptors. Besides uptake from the apoplast, internal Ca^{2+} stores such as organelles or the endoplasmatic reticulum release Ca^{2+} into the cytoplasm through different but specific Ca^{2+} permeable channels, transporters, and exchangers. Elevated $(Ca^{2+})_{cyt}$ and $(Ca^{2+})_{nuc}$ levels are read out by respective sensors, decoders, and transducers including Ca^{2+}-binding proteins and downstream effector proteins, which convert the signature message into appropriate physiological responses. The regulation of $(Ca^{2+})_{cyt}$ and $(Ca^{2+})_{nuc}$ levels is critical to cellular function. The roles of Ca^{2+} signaling during different stages of the development of the symbiosis and during the progression of the infection are slowly unraveling. Mutants with different defects in $(Ca^{2+})_{cyt}$ and $(Ca^{2+})_{nuc}$ elevation in symbiosis formation or pathogenesis give valuable information. The molecular and cellular mechanisms involved in the intracellular accommodation of symbiotic microorganisms in plants are another area of future research.

REFERENCES

Addepalli MK, Fujita Y. Regulatory role of external calcium on *Pythium porphyrae* (Oomycota) zoospore release, development and infection in causing red rot disease of *Porphyra yezoensis* (Rhodophyta). FEMS Microbiol Lett 2002;211:253–257.

Ali R, Ma W, Lemtiri-Chlieh F, Tsaltas D, Leng Q, von Bodman S, Berkowitz GA. Death don't have no mercy and neither does calcium: arabidopsis cyclic nucleotide gated channel2 and innate immunity. Plant Cell 2007;19:1081–1095.

Allen DG, Blinks JR, Prendergast FG. Aequorin luminescence: relation of light emission to calcium concentration – a calcium-independent component. Science 1976;195:996–998.

Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffman T. A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature 2001;411:1053–57.

Allen GJ, Kwak JM, Chu SP, Llopis J, Tsien RY, Harper JF, Schroeder JI. Cameleon calcium indicator reports cytoplasmic calcium dynamics in Arabidopsis guard cells. Plant J 1999;19:735–747.

Allen GJ, Muir SR, Sanders D. Release of Ca^{2+} from individual plant vacuoles by both InsP(3) and cyclic ADP-ribose. Science 1995;268:735–37.

Anil VS, Rao KS. Calcium-mediated signal transduction in plants: a perspective on the role of Ca^{2+} and CDPKs during early plant development. J Plant Physiol 2001;158:1237–1256.

Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, et al. The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. Plant Physiol 2006;142:265–279.

Atkinson MM, Midland SL, Sims JJ, Keen NT. Syringolide 1 triggers Ca^{2+} influx, K^+ efflux, and extracellular alkalization in soybean cells carrying the disease-resistance gene *Rpg4*. Plant Physiol 1996;112:297–302.

Badminton MN, Kendall JM, Rembold CM, Campbell AK. Current evidence suggests independent regulation of nuclear calcium. Cell Calcium 1998;23:79–86.

Batistič O, Kudla J. Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. Planta 2004;219:915–924.

Bécard G, Kosuta S, Tamasloukht M, Séjalon-Delmas N, Roux C. Partner communication in the arbuscular mycorrhizal interaction. Can J Bot 2004;82:1186–1197.

Blume B, Nürnberger T, Nass N, Scheel D. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defence in parsley. Plant Cell 2000;12:1425–1440.

Bonfante P, Genre A. Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective. Trends Plant Sci 2008;13:292–298.

Bonfante P, Requena N. Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol 2011;14:451–457.

Borsics T, Webb D, Andeme-Ondzighi C, Staehelin LA, Christopher DA. The cyclic nucleotide-gated calmodulin-binding channel At-CNGC10 localizes to the plasma membrane and influences numerous growth responses and starch accumulation in *Arabidopsis thaliana*. Planta 2007;225:563–573.

Bourque S, Binet MN, Ponchet M, Pugin A, Lebrun-Garcia A. Characterization of the cryptogein binding sites on plant plasma membranes. J Biol Chem 1999;274:34699–34705.

Bourque S, Ponchet M, Binet MN, Ricci P, Pugin A, Lebrun-Garcia A. Comparison of binding properties and early biological effects of elicitins in tobacco cells. Plant Physiol 1998;118:1317–1326.

Bowler C, Fluhr R. The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant Sci 2000;5:241–246.

Breuninger M, Requena N. Recognition events in AM symbiosis: analysis of fungal gene expression at the early appressorium stage. Fungal Genet Biol 2004;41:794–804.

Buhr TL, Dickman MB. Gene expression analysis during conidial germ-tube and appressorium development in *Colletotrichum trifoli*. Appl Env Microbiol 1997;63:2378–2383.

Bush DS. Calcium regulation in plant cells and its role in signalling. Ann Rev Plant Physiol Plant Mol Biol 1995;27:953–967.

Camehl I, Drzewiecki C, Vadassery J, Shahollari B, Sherameti I, Forzani C, Munnik T, Hirt H, Oelmüller R. The OXI1 Kinase Pathway Mediates *Piriformospora indica*-Induced Growth Promotion in Arabidopsis. PLoS Pathog 2011;7(5):e1002051.

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmüller R. Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 2010;185:1062–1073.

Chabaud M, Genre A, Sieberer BJ, Faccio A, Fournier J, Novero M, Barker DG, Bonfante P. Arbuscular mycorrhizal hyphopodia and germinated spore exudates trigger Ca^{2+} spiking in the legume and nonlegume root epidermis. New Phytol 2011;189:347–355.

Charbonneau H, Walsh KA, McCann RO, Prendergast FG, Cormier MJ, Vanaman TC. Amino acid sequence of the calcium-dependent photoprotein aequorin. Biochemistry 1985;4:6762–6771.

Cheng SH, Willmann MR, Chen HC, Sheen J. Calcium signaling through protein kinases. The *Arabidopsis* calcium-dependent protein kinase gene family. Plant Physiol 2002;129:469–485.

Chico JM, Raices M, Tellez-Inon MT, Ulloa RM. A calcium-dependent protein kinase is systemically induced upon wounding in tomato plants. Plant Physiol 2002;128:256–270.

Choi J, Kim Y, Kim S, Park J, Lee YH. MoCRZ1, a gene encoding a calcineurin-responsive transcription factor, regulates fungal growth and pathogenicity of *Magnaporthe oryzae*. Fungal Genet Biol 2009a;46:243–254.

Choi J, Kim Y, Lee YH. Functional analysis of MCNA, a gene encoding a catalytic subunit of calcineurin, in the rice blast fungus *Magnaporthe oryzae*. J Microbiol Biotechnol 2009b;19:11–16.

Choi J, Kim KS, Rho HS, Lee YH. Differential roles of the phospholipase C genes in fungal development and pathogenicity of *Magnaporthe oryzae*. Fungal Genet Biol 2011;48:445–455.

Christopher DA, Borsics T, Yuen CY, Ullmer W, Andeme-Ondzighi C, Andres MA, Kang BH, Staehelin LA. The cyclic nucleotide gated cation channel AtCNGC10 traffics from the ER via Golgi vesicles to the plasma membrane of *Arabidopsis* root and leaf cells. BMC Plant Biol 2007;19:7.

Chung E, Park JM, Oh SK, Joung YH, Lee S, Choi D. Molecular and biochemical characterization of the *Capsicum annuum* calcium-dependent protein kinase 3 (CaCDPK3) gene induced by abiotic and biotic stresses. Planta 2004;220:286–295.

Clapham DE. Calcium signaling. Cell 2007;131:1047–1058.

Dangl JL, Dietrich RA, Richberg MH. Death don't have mercy: cell death programs in plant–microbe interactions. Plant Cell 1996;8:1793–1807.

Dangl JL, Jones JDG. Plant pathogens and integrated defence responses to infection. Nature 2001;411:826–833.

Deshmukh S, Hückelhoven R, Schäfer P, Imani J, Sharma M, Weiss M, Waller F, Kogel KH. The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci U S A 2006;103:18450–18457.

Dietrich RA, Delaney TP, Uknes SJ, Ward ER, Ryals JA, Dangl JL. *Arabidopsis* mutants simulating disease resistance response. Cell 1994;77:565–577.

Dietrich A, Mayer JE, Hahlbrock K. Fungal elicitor triggers rapid, transient, and specific protein phosphorylation in parsley cell suspension cultures. J Biol Chem 1990;265:6360–6368.

Dixon RA, Harrison MJ, Lamb CJ. Early events in the activation of plant defense responses. Annu Rev Phytopathol 1994;32:479–501.

Dodd AN, Kudla J, Sanders D. The language of calcium signaling. Annu Rev Plant Biol 2010;61:593–620.

Ebel J. Oligoglucoside elicitor-mediated activation of plant defence. Bioessays 1995;20:569–576.

Ebel J, Bhagwat AA, Cosio EG, Feger M, Kissel U, Mithöfer A, Waldmüller T. Elicitor-binding proteins and signal transduction in the activation of a phytoalexin defense response. Can J Bot 1995;73:506–510.

Emmanouilidou E, Teschemacher AG, Pouli AE, Nicholls LI, Seward EP, Rutter GA. Imaging Ca^{2+} concentration changes at the secretory vesicle surface with a recombinant targeted cameleon. Curr Biol 1999;9:915–918.

Ehrhardt DW, Wais R, Long SR. Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. Cell 1996;85:673–681.

Felix G, Grosskopf DG, Regenass M, Boller T. Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells. Proc Natl Acad Sci U S A 1991;88:8831–8834.

Finkler A, Ashery-Padan R, Fromm H. CAMTAs: Calmodulin-binding transcription activators from plants to human. FEBS Lett 2007;581:3893–3898.

Foissner I, Wendehenne D, Longteraals P, Durner J. Technical advance: *in vivo* imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J 2000;23:817–824.

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, et al. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 2003;422:442–446.

Gadella TWJ, Van Der Krogt GNM, Bisseling T. GFP-based FRET microscopy in living plant cells. Trends Plant Sci 1999;4:287–291.

Garcia-Brügger A, Lamotte O, Vandelle E, Bourque S, Lecourieux D, Poinssot B, Wendehenne D, Pugin A. Early signaling events induced by elicitors of plant defenses. Mol Plant–Microbe Inter 2006;19:711–724.

Gelli A, Higgins VJ, Blumwald E. Activation of plant plasma membrane Ca^{2+}-permeable channels by race-specific fungal elicitors. Plant Physiol 1997;113:269–279.

Genre A, Chabaud M, Faccio A, Barker DG, Bonfante P. Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortext of both *Medicago truncatula* and *Daucus carota*. Plant Cell 2008;20:1407–1420.

Genre A, Chabaud M, Timmers T, Bonfante P, Barker DG. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. Plant Cell 2005;17:3489–3499.

Gerber IB, Zeidler D, Durner J, Dubery IA. Early perception responses of *Nicotiana tabacum* cells in response to lipopolysaccharides from *Burkholderia cepacia*. Planta 2004;218:647–657.

Gleason C, Chaudhuri S, Yang TB, Munoz A, Poovaiah BW, Oldroyd GED. Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. Nature 2006;441:1149–1152.

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J. The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J 2000;23:441–450.

Groth M, Takeda N, Perry J, Ushida H, Dräxl S, Brachmann A, et al. *NENA*, a *Lotus japonicus* homolog of *Sec13*, is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development. Plant Cell 2010;22:2509–2526.

Guo FQ, Okamoto M, Crawford NM. Identification of a plant nitric oxide synthase gene involved in hormonal signalling. Science 2003;302:100–103.

Gutjahr C, Novero M, Guether M, Montanari O, Udvardi M, Bonfante P. Presymbiotic factors released by the arbuscular mycorrhizal fungus *Gigaspora margarita* induce starch accumulation in *Lotus japonicus* roots. New Phytol 2009;183:53–61.

Harmon AC, Gribskov M, Harper JF. CDPKs: a kinase for every Ca^{2+} signal? Trends Plant Sci 2000;5:154–159.

Harper JF, Breton G, Harmon A. Decoding Ca^{2+} signals through plant protein kinases. Annu Rev Plant Biol 2004;55:263–288.

Harrison MJ. Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 2005;59:19–42.

Heath MC. The first touch. Physiol Mol Plant Pathol 2000;56:49–50.

Hetherington AM, Brownlee C. The generation of Ca^{2+} signals in plants. Annu Rev Plant Biol 2004;55:401–427.

Horváth B, Yeun LH, Domonkos A, Halász G, Gobbato E, Ayaydin F, et al. *Medicago truncatula* IPD3 is a member of the common symbiotic signaling pathway required for rhizobial and mycorrhizal symbioses. Mol Plant–Microbe Inter 2011;24(11):1345–58.

Hu X, Neill SJ, Yang Y, Cai W. Fungal elicitor Pep-25 increases cytosolic calcium ions, H2O2 production and activates the octadecanoid pathway in *Arabidopsis thaliana*. Planta 2009;229:1201–1208.

Huang FY, Philosoph-Hadas S, Meir S, Callaham DA, Sabato R, Zelcer A, Hepler PK. Increases in cytosolic Ca^{2+} in parsley mesophyll cells correlate with leaf senescence. Plant Physiol 1997;115:51–60.

Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D. Elicitor-stimulated ion fluxes and O_2 from the oxidative burst are essential components in triggering defence gene activation and phytoalexin synthesis in parsley. Proc Natl Acad Sci U S A 1997;94:4800–4805.

Jackson SL, Heath IB. Roles of calcium ions in hyphal tip growth. Microbiol Rev 1993;57:367–382.

Jarosch B, Kogel KH, Schaffrath U. The ambivalence of the barley Mlo locus: mutations conferring resistance against powdery mildew (*Blumeria graminis* f.sp. *hordei*) enhance susceptibility to the rice blast fungus *Magnaporthe grisea*. Mol Plant–Microbe Inter 1999;12:508–514.

Jeworutzki E, Rob M, Roelfsema G, Anschütz U, Krol E, Theo J, Elzenga M, Felix G, Boller T, Hedrich R, Becker D. Early signaling through the *Arabidopsis* pattern recognition receptors FLS2 and EFR involves Ca^{2+}-associated opening of plasma membrane anion channels. Plant J 2010;62:367–378.

Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A, Trewavas A. Circadian oscillations of cytosolic and chloroplastic free calcium in plants. Science 1995;269:1863–1865.

Johnson JM, Nongbri PL, Sherameti I, Oelmüller R. Calcium signaling and cytosolic calcium measurements in plants. Endocyt Cell Res 2011a;21:64–76.

Johnson JM, Sherameti I, Ludwig A, Nongbri PL, Sun C, Varma A, Oelmüller R. Protocols for *Arabidopsis thaliana* and *Piriformospora indica* co-cultivation – a model system to study plant beneficial traits. Endocyt Cell Res 2011b;21:101–113.

Johnson JM, Oelmüller R. Mutualism or parasitism: life in an unstable continuum. What can we learn from the mutualistic interaction between *Piriformospora indica* and *Arabidopsis thaliana*? Endocyt Cell Res 2009;19:81–110.

Jonak C, Okresz L, Bogre L, Hirt H. Complexity, cross talk and integration of plant MAP kinase signaling. Curr Opin Plant Biol 2002;5:415–424.

Karandashov V, Nagy R, Wegmüller S, Amrhein N, Bucher M. Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci U S A 2004;101:6285–6290.

Kawano T, Muto S. Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco cell suspension culture. J Exp Bot 2000;51:685–693.

Kendall JM, Badminton MN, Dormer RL, Campbell AR. Changes in free calcium in the endoplasmic reticulum of living cells detected using targeted aequorin. Anal Biochem 1994;221:173–181.

Kim YK, Li D, Kolattukudy PE. Induction of Ca^{2+}-calmodulin signaling by hard-surface contact primes *Colletotrichum gloeosporioides* conidia to germinate and form appressoria. J Bacteriol 1998;180:5144–5150.

Kim MC, Panstruga R, Elliott C, Muller J, Devoto A, Yoon HW, et al. Calmodulin interacts with MLO protein to regulate defence against mildew in barley. Nature 2002;416:447–451.

Kistner C, Winzer T, Pitzschke A, Mulder L, Sato S, Kaneko T, et al. Seven Lotus japonicus genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. Plant Cell 2005;17:2217–2229.

Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, et al. Nitric oxide and salicylic acid signalling in plant defences. Proc Natl Acad Sci U S A 2000;97:8849–8855.

Klüsener B, Young JJ, Murata Y, Allen GJ, Mori IC, Hugouvieux V, Schroeder JI. Convergence of calcium signalling pathways of pathogenic elicitors and abscisic acid in *Arabidopsis* guard cells. Plant Physiol 2002;130:2152–2163.

Knight MR, Campbell AK, Smith SM, Trewavas AJ. Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature 1991;352:524–526.

Knight H, Trewavas AJ, Knight MR. Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell 1996;3:489–503.

Knight H, Trewavas AJ, Knight MR. Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J 1997;12:1067–1078.

Kosuta S, Chabaud M, Lougnon G, Gough C, Denarie J, Barker DG, Becard G. A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots of *Medicago truncatula*. Plant Physiol 2003;131:952–962.

Kosuta S, Hazledine S, Sun J, Miwa H, Morris RJ, Downie JA, Oldroyd GED. Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. Proc Natl Acad Sci U S A 2008;105:9823–9828.

Kudla J, Batistic O, Hashimoto K. Calcium signals: the lead currency of plant information processing. Plant Cell 2010;22:541–563.

Kumar J, Hückelhoven R, Beckhove U, Nagarajan S, Kogel KH. A compromised Mlo pathway affects the response of barley to the necrotrophic fungus *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) and its toxins. Phytopathology 2001;91:127–133.

Kuhn H, Küster H, Requena N. Membrane steroid-binding protein 1 induced by a diffusible fungal signal is critical for mycorrhization in *Medicago truncatula*. New Phytol 2010;185:716–733.

Kurusu T, Yagala T, Miyao A, Hirochika H, Kuchitsu K. Identification of a putative voltage-gated Ca2+ channel as a key regulator of elicitor-induced hypersensitive cell death and mitogen-activated protein kinase activation in rice. Plant J 2005;42:798–809.

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, et al. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J 2003;22:2623–2633.

Lamb CJ, Lawton MA, Dixon RA. Signal and transduction mechanisms for activation of plant defenses against microbial attack. Cell 1989;56:215–224.

Lamotte O, Gould K, Lecourieux D, Sequeira-Legrand A, Lebrun-Garcia A, Durner J, Pugin A, Wendehenne D. Analysis of nitric oxide signalling functions in tobacco cells challenged by the elicitor cryptogein. Plant Physiol 2004;135:516–529.

Lecourieux D, Mazars C, Pauly N, Ranjeva R, Pugin A. Analysis and effects of cytosolic free calcium elevation in response to elicitors in *Nicotiana plumbaginifolia* cells. Plant Cell 2002;14:2627–2641.

Lecourieux D, Ranjeva R, Pugin A. Calcium in plant defence-signaling pathways. New Phytol 2006;171:249–269.

Lecourieux-Ouaked F, Pugin A, Lebrun-Garcia A. Phosphoproteins involved in the signal transduction of cryptogein, an elicitor of defence reactions in tobacco. Mol Plant–Microbe Inter 2000;13:821–829.

Lee J, Klessig DF, Nürnberger T. A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. Plant Cell 2001;13:1079–1093.

Lee J, Rudd JJ. Calcium-dependent protein kinases: versatile plant signaling components necessary for pathogen defence. Trends Plant Sci 2002;7:97–98.

Lee SC, Lee YH. Calcium/calmodulin-dependent signaling for appressorium formation in the plant pathogenic fungus *Magnaporthe grisea*. Mol Cells 1998;8:698–704.

Lee YC, Johnson JM, Chien CT, Sun C, Cai D, Lou B, Oelmüller R, Yeh KW. Growth promotion of Chinese cabbage and *Arabidopsis* by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin. Mol Plant–Microbe Inter 2011;24(4):421–31.

Levine A, Pennell R, Palmer R, Lamb CJ. Calcium-mediated apoptosis in a plant hypersensitive response. Curr Biol 1996;6:427–437.

Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, et al. A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 2004;303:1361–1364.

Link VL, Hofmann MG, Sinha AK, Ehness R, Strand M, Roitsch T. Biochemical evidence for the activation of distinct subsets of mitogen-activated protein kinases by voltage and defence-related stimuli. Plant Physiol 2002;128:271–281.

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W. Calmodulins and calcineurin B–like proteins: calcium sensors for specific signal response coupling in plants. Plant Cell 2002;14:389–400.

Ma W, Berkowitz GA. The grateful dead: calcium and cell death in plant innate immunity. Cell Microbiol 2007;9(11):2571–2585.

McAinsh MR, Pittman JK. Shaping the calcium signature. New Phytol 2009a;181:275–94.

McAinsh MR, Pittman JL. Shaping the calcium signature. New Phytol 2009b;181:275–294.

McAinsh MR, Webb A, Taylor JE, Hetherington AM. Stimulus-induced oscillations in guard cell cytosolic free calcium. Plant Cell 1995;7:1207–1219.

Maillet F, Poinsot V, Andre O, Puech-Pages V, Haouy A, Gueunier M, et al. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 2011;69:58–63.

Malhó R, Moutinho A, van der Luit A, Trewavas AJ. Spatial characteristics calcium signalling: the calcium wave as a basic unit in plant cell calcium signalling. Philos Trans R Soc Lond Ser B 1998;353:1463–1473.

Mithöfer A, Ebel J, Bhagwat AA, Boller T, Neuhaus-Url G. Transgenic aequorin monitors cytosolic transients in soybean cells challenged with β-glucan or chitin elicitors. Planta 1999;207:566–574.

Mithöfer A, Mazars C. Aequorin-based measurements of intracellular Ca^{2+}-signatures in plant cells. Biol Proced Online 2002;4:105–118.

Miwa H, Sun J, Oldroyd GE, Downie JA. Analysis of Nod-factor-induced calcium signaling in root hairs of symbiotically defective mutants of *Lotus japonicus*. Mol Plant–Microbe Inter 2006;19:914–923.

Miyawaki A, Greisbeck O, Heim R, Tsien RY. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. Proc Natl Acad Sci U S A 1999;96:2135–2140.

Miyawaki A, Llopis J, Heim R, McCaffery JM, Adamas JA, Ikura M, Tsien RY. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature 1997;388:882–887.

Montero M, Alonso MT, Carnicero E, Cuchillo-Ibáñez I, Albillos A, García AG, García-Sancho J, Alvarez J. Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion. Nat Cell Biol 2000;2:57–61.

Mori IC, Schroeder JI. Reactive oxygen species activation of plant Ca^{2+} channels: a signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. Plant Physiol 2004;135:702–708.

Moyen C, Hammond-Kosack JE, Jones J, Knight MR, Johannes E. Systemin triggers an increase of cytoplasmic calcium in tomato mesophyll cells: Ca^{2+} mobilization from intra- and extra-cellular compartments. Plant Cell Environ 1998;21:1101–1111.

Müller J, Staehelin C, Xie Z-P, Neuhaus-Url G, Boller T. Nod factors and chitooligomers elicit an increase in cytosolic calcium in aequorin-expressing soybean cells. Plant Physiol 2000;124:733–740.

Musicki B, Kishi Y, Shimomura O. Structure of the functional part of photoprotein aequorin. Chem Commun 1986;1986:1566–1568.

Navazio L, Moscatiello R, Genre A, Novero M, Baldan B, Bonfante P, Mariani P. A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. Plant Physiol 2007;144:673–681.

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT. Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot 2002;53:1237–1247.

Ng CK-Y, Carr K, McAinsh MR, Powell B, Hetherington AM. Drought induced guard cell signal transduction involves sphingosine-1-phosphate. Nature 2001;410:596–599.

Nguyen QB, Kadotani N, Kasahara S, Tosa Y, Mayama S, Nakayashiki H. Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNAsilencing system. Mol Microbiol 2008;68:1348–1365.

Nürnberger T, Nennstiel D, Jabs T, Sacks W, Hahlbrock K, Scheel D. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. Cell 1994;78:449–460.

Nürnberger T, Scheel D. Signal transmission in the plant immune response. Trends Plant Sci 2001;6:372–379.

Oelmüller R, Sherameti I, Tripathi S, Varma A. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 2009;49:1–17.

Ogasawara Y, Kaya H, Hiraoka G, Yumoto F, Kimura S, Kadota Y, et al. Synergistic activation of the *Arabidopsis* NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. J Biol Chem 2008;283:8885–8892.

Olah B, Briere C, Becard G, Denarie J, Gough C. Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1DMI2 signaling pathway. Plant J 2005;44:195–207.

Oldroyd GED, Downie JA. Coordination of nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 2008;59:519–546.

Panstruga R, Schulze-Lefert P. Corruption of host seven-transmembrane proteins by pathogenic microbes: a common theme in animals and plants? Microbes Infect 2003;5:429–437.

Parniske M. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 2008;6:763–775.

Paszkowski U. A journey through signaling in arbuscular mycorrhizal symbioses. New Phytol 2006;172:35–46.

Pauly N, Knight MR, Thuleau P, Graziana A, Muto S, Ranjeva R, Mazars R. The nucleus together with the cytosol generates patterns of specific cellular calcium signatures in tobacco suspension culture cells. Cell Calcium 2001;30:413–421.

Peck SC, Nühse TS, Hess D, Iglesias A, Meins F, Boller T. Directed proteomics identifies a plant-specific protein rapidly phosphorylated in response to bacterial and fungal elicitors. Plant Cell 2001;13:1467–1475.

Persechini A, Moncrief ND, Kretsinger RH. The EF-hand family of calcium-modulated proteins. Trends Neurosci 1989;12:462–467.

Plieth C. Calcium: just another regulator in the machinery of life? Ann Bot (London) 2005;96:1–8.

Pumplin N, Harrison MJ. Live-cell imaging reveals periarbuscular membrane domains and organelle location in *Medicago truncatula* roots during arbuscular mycorrhizal symbiosis. Plant Physiol 2009;151:809–819.

Ranf S, Grimmera J, Pöschl Y, Pecher P, Chinchilla D, Scheel D, Lee J. Defense-related calcium signaling mutants uncovered via a quantitative high-throughput screen in *Arabidopsis thaliana*. Mol Plant 2011. DOI: DOI: 10.1093/mp/ssr064.

Reddy ASN. Calcium: silver bullet in signaling. Plant Sci 2001;160:381–404.

Reddy VS, Reddy ASN. Proteomics of calcium-signaling components in plants. Phytochemistry 2004;65:1745–1776.

Romeis T, Piedras P, Zhang S, Klessig DF, Hirt H, Jones JD. Rapid Avr9- and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves. Convergence of resistance gene, elicitor, wound, and salicylic acid responses. Plant Cell 1999;11:273–288.

Romeis T, Piedras P, Jones JD. Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defence response. Plant Cell 2000;12:803–816.

Rudd JJ, Franklin-Tong VE. Unravelling response-specificity in Ca^{2+} signaling pathways in plant cells. New Phytol 2001;151:7–33.

Sanders D, Brownlee C, Harper JF. Communicating with calcium. Plant Cell 1999;11:691–706.

Sanders D, Pelloux J, Brownlee C, Harper JF. Calcium at the crossroads of signaling. Plant Cell 2002;14:S401–417.

Sasabe M, Takeuchi K, Kamoun S, Ichinose Y, Govers F, Toyoda K, Shiraishi T, Yamada T. Independent pathways leading to apoptotic cell death, oxidative burst and defence gene expression in response to elicitin in tobacco cell suspension culture. Eur J Biochem 2000;267:5005–5013.

Shahollari B, Vadassery J, Varma A, Oelmüller R. A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 2007;50(1):1–13.

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R. The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. J Biol Chem 2005;280:2641–2647.

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R. PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 2008;54:428–439.

Shimomura O, Johnson FH. Peroxidized coelenterazine, the active group in the photoprotein aequorin. Proc Natl Acad Sci U S A 1978;75:2611–2615.

Smith SE, Read DJ. Mycorrhizal Symbiosis. 3rd ed. New York, London: Academic Press; 2008. p 787.

Smith FA, Smith SE. Structural diversity in (vesicular)-arbuscular mycorrhizal symbiosis. New Phytol 1997;137:373–388.

Smith SE, Smith SF. Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. Annu Rev Plant Biol 2011;62:227–250.

Song S, Jia Z, Xu J, Zhang Z, Bian Z. N-Butyryl-homoserine lactone, a bacterial quorum-sensing signaling molecule, induces intracellular calcium elevation in *Arabidopsis* root cells. Biochem Biophys Res Commun 2011;414:355–360.

Stäb MR, Ebel J. Effects of Ca^{2+} on phytoalexin induction by fungal elicitor in soybean cells. Arch Biochem Biophys 1987;257:416–423.

Stein E, Molitor A, Kogel KH, Waller F. Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. Plant Cell Physiol 2008;49:1747–1751.

Sun C, Johnson JM, Cai D, Sherameti I, Oelmüller R, Lou B. *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. J Plant Physiol 2010;167:1009–1017.

Takahashi A, Camacho P, Lechleiter JD, Herman B. Measurement of intracellular calcium. Physiol Rev 1999;79:1089–1125.

Tamasloukht M, Sejalon-Delmas N, Kluever A, Jauneau A, Roux C, Becard G, Franken P. Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. Plant Physiol 2003;131:1468–1478.

Tavernier E, Wendehenne D, Blein JP, Pugin A. Involvement of free calcium in action of cryptogein, a proteinaceous elicitor of hypersensitive reaction in tobacco cells. Plant Physiol 1995;109:1025–1031.

Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen L, Miwa H, et al. Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. Nature 2006;441:1153–1156.

Torres MA, Dangl JL, Jones JD. Arabidopsis *gp91phox* homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defence response. Proc Natl Acad Sci U S A 2002;99:517–522.

Tsuji FI, Inouye S, Goto T, Sakaki Y. Site-specific mutagenesis of the calcium-binding photoprotein aequorin. Proc Natl Acad Sci U S A 1986;83:8107–8111.

Uhm KH, Ahn IP, Kim S, Lee YH. Calcium/calmodulin-dependent signaling for prepenetration development in *Colletotrichum gloeosporioides*. Phytopathology 2003;93:82–87.

Vadassery J, Oelmüller R. Calcium signaling in pathogenic and beneficial plantmicrobe interactions what can we learn from the interaction between *Piriformospora indica* and *Arabidopsis thaliana*. Plant Signal Behav 2009;4:1024–1027.

Vadassery J, Ranf S, Drewiecki C, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R. A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. Plant J 2009;59:193–206.

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B, et al. The role of auxins and cytokinins in the mutualistic interaction between *Arabidopsis* and *Piriformospora indica*. Mol Plant–Microbe Inter 2008;21(10):1371–1383.

Vahabi K, Johnson JM, Drzewiecki C, Oelmüller R. Fungal staining tools to study the interaction between the beneficial endophyte *Piriformospora indica* with *Arabidopsis thaliana* roots. Endocyt Cell Res 2011;21:77–88.

Vandelle E, Poinssot B, Wendehenne D, Bentéjac M, Pugin A. Integrated signalling network involving calcium, nitric oxide, and active oxygen species but not mitogen-activated protein kinases in BcPG1-elicited grapevine defences. Mol Plant–Microbe Inter 2006;19:429–440.

Varma A, V S, Sudha N, Sahay B, Butehorn B, Franken P. *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 1999;65(6):2741–2744.

Varma A, Singh A, Sudha S, Sahay N, Sharma J, Roy A, et al. *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Mycota IX, Bock B, editors. Springer Series. Berlin: Springer-Verlag; 2001. p 123–150.

Vidhyasekaran P. *Fungal Pathogenesis in Plants and Crops: Molecular Biology and Host Defense Mechanisms*. 2nd ed. Florida, London, New York: CRC Press; 2008.

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, et al. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci U S A 2005;102(38):13386–13391.

Webb AAR, McAinsh MR, Taylor JE, Hetherington AM. Calcium ions as intracellular second messengers in higher plants. Adv Bot Res 1996;22:45–96.

Weidmann S, Sanchez L, Descombin J, Chatagnier O, Gianinazzi S, Gianinazzi-Pearson V. Fungal elicitation of signal transduction-related plant genes precedes mycorrhiza establishment and requires the dmi3 gene in *Medicago truncatula*. Mol Plant–Microbe Inter 2004;17:1385–1393.

Wendehenne D, Durner J, Klessig DF. Nitric oxide: a new player in plant signalling and defence responses. Curr Opin Plant Biol 2004;7:449–455.

Wendehenne D, Lamotte O, Frachisse JM, Barbier-Brygoo H, Pugin A. Nitrate efflux is an essential component of the cryptogein signaling pathway leading to defence responses and hypersensitive cell death in tobacco. Plant Cell 2002;14:1937–1951.

Westheimer FH. Why nature chose phosphates. Science 1987; 235:1173–1178.

White PJ, Broadley MR. Calcium in plants. Ann Bot (Lond) 2003;92:487–511.

White PJ, Davenport RJ. The voltage-independent cation channel in the plasma membrane of wheat roots is permeable to divalent cations and may be involved in cytosolic Ca^{2+} homeostasis. Plant Physiol 2002;130:1386–1395.

Wojtaszek P. Oxidative burst: an early response to pathogen infection. Biochem J 1997;322:681–692.

Xu H, Heath MC. Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus. Plant Cell 1998;10:585–598.

Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathy T, Tuteja N, Saxena AK, Johri AK. A phosphate transporter from a root endophytic fungus *Piriformospora indica* plays a role in the phosphate transfer to the plants. J Biol Chem 2010;285:26532–26544.

Yang T, Poovaiah BW. A calmodulin-binding/CGCG box DNA-binding protein family involved in multiple signalling pathways in plants. J Biol Chem 2002;277:45049–45058.

Yano K, Yoshida S, Müller J, Singh S, Banba M, Vickers K, et al. CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc Natl Acad Sci U S A 2008;105:20540–20545.

Yoshioka K, Moeder W, Kang H-G, Kachroo P, Masmoudi K, Berkowitz G, Klessig DF. The chimeric *Arabidopsis* cyclic nucleotide gated ion channel 11/12 activates multiple pathogen resistance responses. Plant Cell 2006;18:747–763.

Zhang HJ, Fang Q, Zhang ZG, Wang YC, Zheng XB. The role of respiratory burst oxidase homologues in elicitor-induced stomatal closure and hypersensitive response in *Nicotiana benthamiana*. J Exp Bot 2009;60:3109–3122.

Zhang S, Klessig DF. MAPK cascades in plant defence signaling. Trends Plant Sci 2001;6:520–527.

Zhang L, Lu YT. Calmodulin-binding protein kinases in plants. Trends Plant Sci 2003;8:123–127.

Zimmermann S, Nürnberger T, Frachisse JM, Wirtz W, Guern J, Hedrich R, Scheel D. Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defence. Proc Natl Acad Sci U S A 1997;94:2751–2755.

Zuccaro A, Lahrmann U, Güldener U, Langen G, Pfiffi S, Biedenkopf D, et al. Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. PLoS Pathog 2011;7:e1002290. DOI: 10.1371/journal.ppat.1002290.

4.4 Manuscript IV (in preparation)

Growth and Defense - Ca^{2+} signaling in unstable continuum: *Piriformopsora indica*-derived trisaccharide induces intracellular calcium elevation, promotes growth and confers resistance in *Arabidopsis thaliana*

Joy Michal Johnson, Ravikumar Maddula, Michael Reichelt, Sybille Lorenz, Mitsuhiro Matsuo, Rinesh Godfrey, Jyothilakshmi Vadassery, Pyniarlang L. Nongbri, Frank D-Böhmer, Jonathan Gershenzon, Bernd Schneider, Ales Svatos and Ralf Oelmüller

Supplementary informations are kept as separate files in the attached CD.

Growth and Defense - Ca^{2+} signaling in unstable continuum: *Piriformopsora indica*-derived trisaccharide induces intracellular calcium elevation, promotes growth and confers resistance in *Arabidopsis thaliana*

Joy Michal Johnson[1], Ravikumar Maddula[2], Michael Reichelt[2], Sybille Lorenz[2], Mitsuhiro Matsuo[1], Rinesh Godfrey[3], Jyothilakshmi Vadassery[2], Pyniarlang L. Nongbri[1], Frank D-Böhmer[3], Jonathan Gershenzon[2], Bernd Schneider[2], Ales Svatos[2] and Ralf Oelmüller[1*]

(*) corresponding author; email: b7oera@hotmail.de

[1]Institute of General Botany and Plant Physiology, Friedrich-Schiller-Universität Jena, Dornburger Str. 159, 07743 Jena, Germany; [2]Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Str. 8, D-07745 Jena, Germany. [3]Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Hans-Knöll-Str. 2, D-07745 Jena, Germany.

Key words: abiotic stress, *Alternaria brassicae*-toxin, biotic stress, cell wall extract, cytosolic calcium elevation, Pi-504, *Piriformospora indica*, reactive oxygen species, redox responsive transcription factor1

Abbreviations: bMAMP, beneficial microbe-associated molecular pattern; $[Ca^{2+}]_{cyt}$, cytosolic calcium concentration; *pi-cycam*, cytosolic calcium mutant to Pi-504; CWE, cell wall extract; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; WT, wild-type

Running title: Pi-504 induces $[Ca^{2+}]_{cyt}$ elevation, promotes growth and confers resistance in Arabidopsis

Abstract

Piriformospora indica (Pi), an endophytic fungus, colonizes the roots of many plant species including *Arabidopsis thaliana* leading to growth promotion and enhanced seed yield. A Pi-cell wall extract (Pi-CWE) induces cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation and promotes growth, as the fungus does. We purified and characterized the Pi-CWE as a trisaccharide with an accurate mass of 504.1748 Da (Pi-504). Pi-504 induces $[Ca^{2+}]_{cyt}$ elevation in roots of Arabidopsis seedlings and promotes growth in Arabidopsis and tobacco. We screened an EMS mutagenized apoaequorin seedling-population and identified mutants which do not respond to Pi-504 for $[Ca^{2+}]_{cyt}$ elevation and growth promotion. The impaired growth promotion is

evidenced by low biomass, reduced photosynthetic efficiency and downregulation of primary metabolism genes. The mutant is more sensitive to abiotic stress such as salt and oxidative stress, and hypersensitive to the necrotrophic fungus, *Alternaria brassicae*. Therefore, the mutated gene required for $[Ca^{2+}]_{cyt}$ elevation is involved in defense responses. Loss-of-function of gene involved in inducing $[Ca^{2+}]_{cyt}$ elevation, affected the tight regulation of reactive oxygen species (ROS) production, in response to *A. brassicae* infection. Genetic and pharmacological studies show that Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation is upstream of redeox responsive transcription factor 1 (*RRTF1*) which amplifies ROS in Arabidopsis. These results suggest that the *P. indica*-induced $[Ca^{2+}]_{cyt}$ elevation is crucial for growth promotion, and the activation of defense against biotic and abiotic responses. Pi-504-induced Ca^{2+} signaling cascades, repressed the *A. brassicae*-toxin-induced Ca^{2+} signaling cascades which lead to hypersusceptibility of Arabidopsis to *A. brassicae*.

INTRODUCTION

Being sessile in nature, plants must adapt themselves to changing environments by their ability to sense and respond rapidly to a wide variety of biotic and abiotic stimuli. Plants have evolved very effective mechanisms to perceive, transduce and respond to a wide variety of biotic and abiotic signals by modulating intracellular or cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (c.f. Sanders et al., 2002; Lecourieux et al., 2006; Dodd et al., 2010; Oldroyd et al., 2011; Reddy et al., 2011). Ca^{2+}-based signaling systems are composed of a receptor, a system for generating the transient or slow increase in $[Ca^{2+}]_{cyt}$, downstream components that are capable of sensing the increase in $[Ca^{2+}]_{cyt}$, transducing the signature message to targets and other cellular systems responsible for returning $[Ca^{2+}]_{cyt}$ to its prestimulus level (Sanders et al., 2002; Hetherington and Brownlee, 2004). Under resting conditions, $[Ca^{2+}]_{cyt}$ is maintained below 100 nm, 10^4 times less than that in the apoplastic fluid and 10^4 to 10^5 times less than that in different cellular Ca^{2+} stores e.g. vacuoles, endoplasmic reticulum, chloroplast, mitochondria and nucleus resulting in a steep gradient which is essential for signaling process (Lecourieux et al., 2006; Kudla et al., 2010). $[Ca^{2+}]_{cyt}$ changes can be quantitatively measured in plants by a noninvasive recombinant aequorin technology (Knight et al., 1991; 1997).

Plants interact with a wide array of microorganisms, with interactions being beneficial or pathogenic to the plants. $[Ca^{2+}]_{cyt}$ elevation is one of the earliest physiological responses in root and leaf cells in response to pathogenic and symbiotic fungi, and are key determinants for

the development of symbiosis, disease resistance or susceptibility in plants (Garcia-Bruggger et al., 2006; Chabaud et al., 2011). Upon perception of signals from beneficial fungi or/and its beneficial microbe-associated molecular patterns (bMAMPs) and pathogenic fungi or/and its pathogen-associated molecular patterns (PAMPs), $[Ca^{2+}]_{cyt}$ levels transiently increase in their host cells within seconds in a receptor mediated process to activate Ca^{2+} signaling cascades (Blume et al., 2000; Lecourieux et al., 2002; Navazio et al., 2007; Hu et al., 2009; Vadassery et al., 2009). Plants discriminate both the nature and strength of these stimuli to mount an appropriate rapid adaptive response for their survival (Mithöfer and Mazars, 2002). The interplay of Ca^{2+} signaling and their information processing during plant-fungus interactions are the key determinants in the decision either to establish symbiosis or disease resistance/-susceptibility in plants (Lecourieux et al., 2006; Dodd et al., 2010; Kudla et al., 2010).

The beneficial interaction of the root endophyte, *Piriformospora indica* with a multitude of horticulturally and agriculturally important plants as well as model plants including *Arabidopsis thaliana* ultimately leads to growth promotion, increased biomass production and enhanced resistance/tolerance against biotic and abiotic stress (cf. Varma et al., 1999; Peškan-Berghöfer et al., 2004; Sherameti et al., 2005; Waller et al., 2005; Shahollari et al., 2007; Vadassery et al., 2009; Sun et al., 2010; Lee et al., 2011; Johnson et al., 2011b). The fungus can be cultivated axenically on synthetic or complex media without a host (Varma et al., 1999, 2001; Peškan-Berghöfer et al., 2004). The plants benefit from this relationship by reprogramming plant transcriptomes, proteomes and metabolomes that are directly or indirectly involved in phytohormone synthesis and signaling, increased root and shoot growth, increased nutrient uptake, early flowering, enhanced seed production, and protection against drought, salinity and phytopathogens (Waller et al., 2005; Deshmukh et al., 2006; Shahollari et al., 2007; Sherameti et al., 2008; Stein et al., 2008; Vadassery et al., 2008, 2009; Camehl et al., 2010, 2011; Sun et al., 2010; Yadav et al., 2010; Lee et al., 2011; Zuccaro et al., 2011; Jacobs et al., 2011) . The modulation of gene expressions, proteins and metabolites helps both partners to keep the interaction mutually beneficial. As the fungus can colonize the roots of most of the plant species tested so far, the interaction between the symbiotic partners should be based on general recognition and signaling processes (Oelmüller et al., 2009). Ca^{2+} signaling plays a major role in the endosymbiotic interaction between *P. indica* and *A. thaliana* (Vadassery et al., 2009; Johnson et al., 2011a; Nongbri et al., 2012). One of the earliest signaling events in this beneficial interaction is a transient increase of $[Ca^{2+}]_{cyt}$

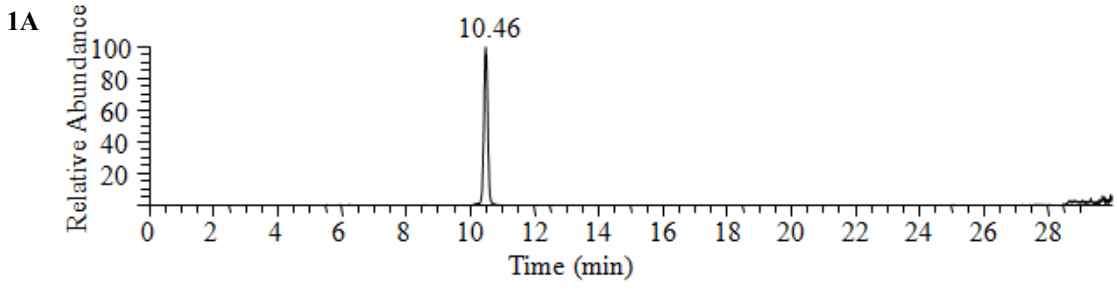
followed by a nuclear Ca^{2+} response (Vadassery et al., 2009). A heat stable cell wall extract (CWE) from the fungus induces $[Ca^{2+}]_{cyt}$ elevation and also promotes the growth of Arabidopsis seedlings as the fungus does. The CWE and the fungus induce similar sets of genes in Arabidopsis roots, which also include genes with Ca^{2+} signaling-related functions (Vadassery et al., 2009). However, the active component in the CWE and the early signaling events in the plant cell leading to growth promotion and defense responses in this beneficial plant/microbe interaction are mostly not known.

Here, we report the purification and characterisation of a trisaccharide from *P. indica*-CWE (Pi-504), which is responsible for $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis roots, and growth promotion in Arabidopsis and tobacco, as the fungus does. We isolated an Arabidopsis mutant, *pi-cycam* (Pi-504-induced cytosolic calcium elevation mutant) which fails to induce $[Ca^{2+}]_{cyt}$ elevation, and also impairs in growth promotion, and tolerance to biotic and abiotic stress. Loss-of-function of gene involved in Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation, affected the tight regulation of reactive oxygen species (ROS) production, in response to *A. brassicae* infection. Genetic and pharmacological studies further showed that Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation is upstream of redox responsive transcription factor 1 (*RRTF1*), which amplifies ROS in Arabidopsis.

RESULTS

A trisaccharide is the active component in the cell wall extract of *P. indica*

The crude Pi-CWE which induces $[Ca^{2+}]_{cyt}$ elevation in roots and also promotes growth in Arabidopsis (Vadassery et al., 2009), was further purified using HPLC and UPLC as described in the methods. The biologically active compound in Pi-CWE was purified as a trisaccharide ($C_{18}H_{32}O_{16}$) with *m/z* (mass by charge ratio) of 505.1748 (Pi-504; (Figures 1A-B). After 1 min of background measurement, the Pi-504 treated Arabidopsis roots induced a rapid and transient increase in the $[Ca^{2+}]_{cyt}$ (Figure 2A). After a lag phase of 15-20 ± 5 second,



Chromatogram of Pi-504; RT=1 to 30min

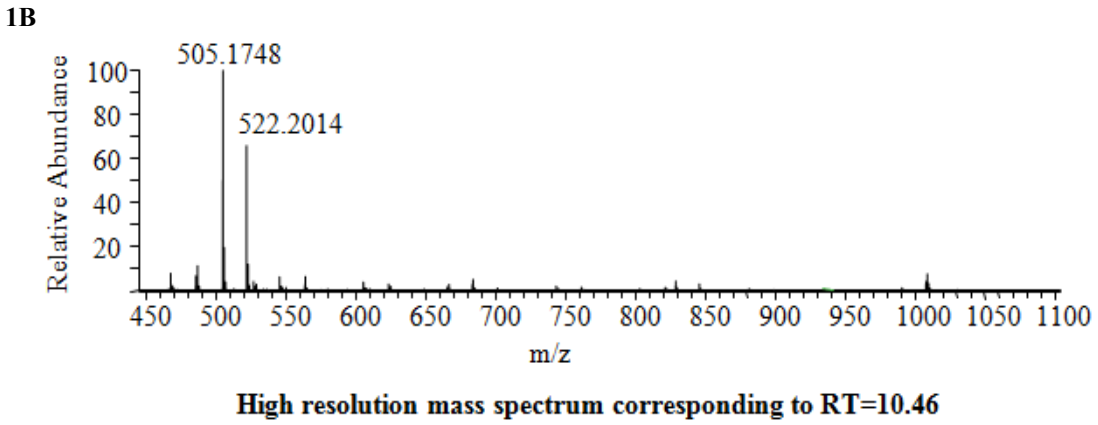


Figure 1. Purification of Pi-504 from Pi-CWE. Pi-504 was purified from the Pi-CWE as described in the methods based on its property to induce $[Ca^{2+}]_{cyt}$ elevation and growth promotion in Arabidopsis seedlings. Pi-504 has an accurate molecular mass of m/z (mass by charge ratio) 505.1748. The chromatogram of Pi-504; RT=10.46 (A) and the high resolution LC-MS spectrum corresponding to RT=10.46 min (B) are shown.

the $[Ca^{2+}]_{cyt}$ level begins to rise and a sharp Ca^{2+} peak of 507 ± 6 nM at 40 ± 5 s was observed (Figure 2A). This transient Ca^{2+} peak was followed by slow and gradual decrease of $[Ca^{2+}]_{cyt}$ level and did not reach to the base level even after 80 min (Figure 2B). The magnitude of $[Ca^{2+}]_{cyt}$ response was dose-dependent as the increasing or decreasing concentration of Pi-CWE increases or decreases the $[Ca^{2+}]_{cyt}$ level (Figure 2C). The strong acid hydrolysis of Pi-504 cleaved the trisaccharide into glucose and galactose (data not shown) and did not induce $[Ca^{2+}]_{cyt}$ elevation in roots (Figure 2D). Pi-504 also promoted the growth of Arabidopsis and

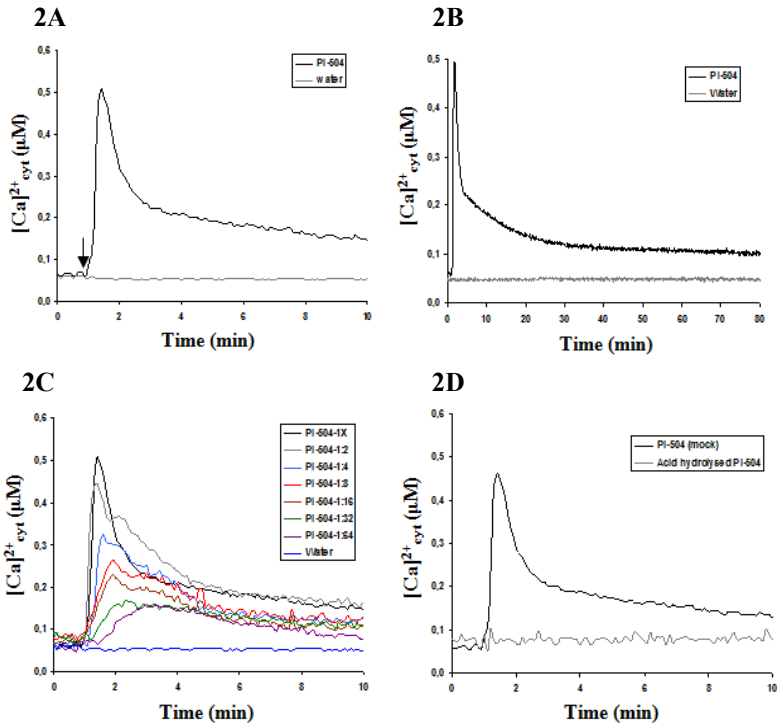


Figure 2. Pi-504 induces $[Ca^{2+}]_{cyt}$ elevation in *A. thaliana* seedlings expressing cytosolic aequorin. Mean (± SE, n=40) $[Ca^{2+}]_{cyt}$ elevation in roots of 18-day old seedlings challenged with 50 µl of Pi-504 as described in 'methods' for 10 min. The arrow indicates the time (1 min) of addition of the stimuli/water (**A**). Prolonged $[Ca^{2+}]_{cyt}$ elevation after treating the roots with 50 µl Pi-504 for 80 min (**B**). Dose dependent increase in $[Ca^{2+}]_{cyt}$ elevation in Pi-504 treated seedling roots (**C**). Acid hydrolysis of Pi-504 completely inhibited $[Ca^{2+}]_{cyt}$ elevation (**D**). In all the experiments, sterile water was used as control and gave background readings.

tobacco seedlings when they were grown on MS medium amended with Pi-504 (Figures 3A-C), although the growth response to Pi-504 was significantly less in both Arabidopsis and tobacco seedlings compared to the fungus-induced growth promotion (Figures 3A-F). These results clearly demonstrate that Pi-504 is a bMAMP which induces $[Ca^{2+}]_{cyt}$ elevation in roots and promotes the growth of Arabidopsis and tobacco seedlings as the fungus does.

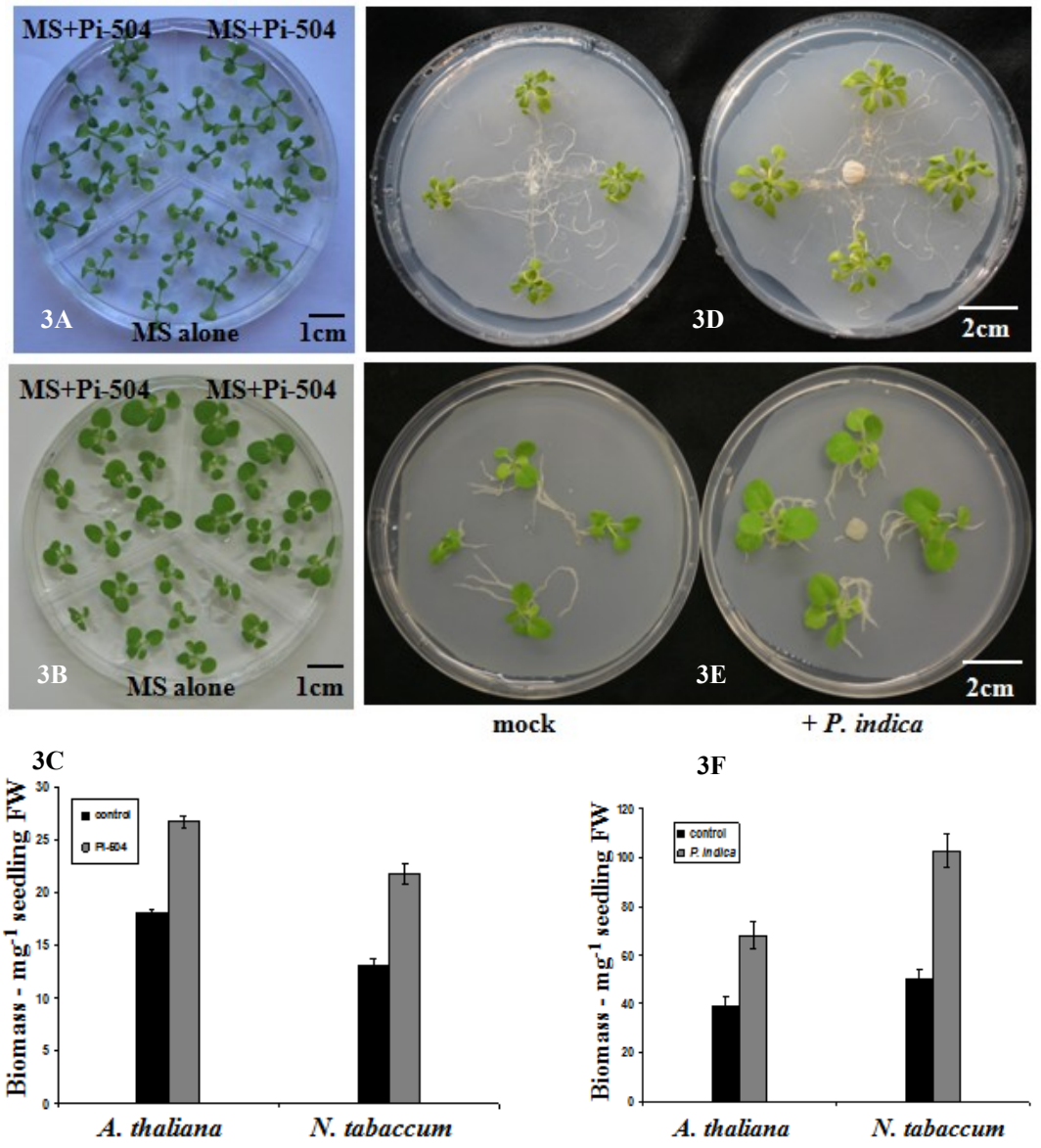


Figure 3. Pi-504 promotes the growth of Arabidopsis and tobacco seedlings. Arabidopsis (WT-aequorin - pMAQ2) and tobacco were grown on MS medium supplemented with 3% of Pi-504 at a strength used to measure $[Ca^{2+}]_{cyt}$ elevation for 14 days. Pi-504 promoted the growth of Arabidopsis seedling **(A)** and tobacco seedling **(B)**. The growth promotion was quantified as biomass on fresh weight (FW) basis **(C)**. Mock treatment was done with sterile water. The growth promotion by *P. indica* is shown in Arabidopsis **(D)** and tobacco **(E)** and was quantified as biomass in FW (**F**). Mock treatment was done with KM plug. The pictures are representative and values are mean ± SE of four independent experiments with N = 50 per treatment per experiment.

Isolation of $[Ca^{2+}]_{cyt}$ mutants nonresponsive to Pi-504

To identify mutants defective in Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation in the early symbiotic interaction of *P. indica* with Arabidopsis seedlings, we screened 0.4 and 0.2% (v/v) EMS-mutagenized M_2 populations of the transgenic Arabidopsis expressing cytosolic apoaequorin in Col-0 background (pMAQ2) with Pi-504 treatment. Approximately 70% of the roots from 16-18-day old individual M_2 seedlings (24 seedlings from individual M_1 plants) grown on Hoagland (HL) medium was dissected and used to measure $[Ca^{2+}]_{cyt}$ elevation (the detailed protocol: Johnson et al., 2011a) and Pi-504 was used as the stimulus. The mutant seedlings were rescued for seed collection. Out of 2,40,000 M_2 seedlings of 10,000 individual M_1 lines screened, 8 independent putative mutants completely failed to induce $[Ca^{2+}]$ elevation in response to Pi-504 (Figure 4A). The mutants were transferred to soil and M_3 seeds were propagated. Seedlings from 4 independent mutants e.g. *pi-cycam1, pi-cycam2, pi-cycam3* and *pi-cycam4* (cytosolic calcium mutant *to* Pi-504) did not show any $[Ca^{2+}]_{cyt}$ elevation to Pi-504 and are thus homozygous (Supplemental Material 1). *pi-cycam1* and *pi-cycam2* were used for further studies as they did not show any visible growth phenotype compared to WT (Figure 4B). To determine the inheritance pattern of *Pi-CYCAM*, *pi-cycam1* and *pi-cycam2* were backcrossed to their ancestor WT (Col-0) as well as outcrossed to WT (La-*er*) ecotype and

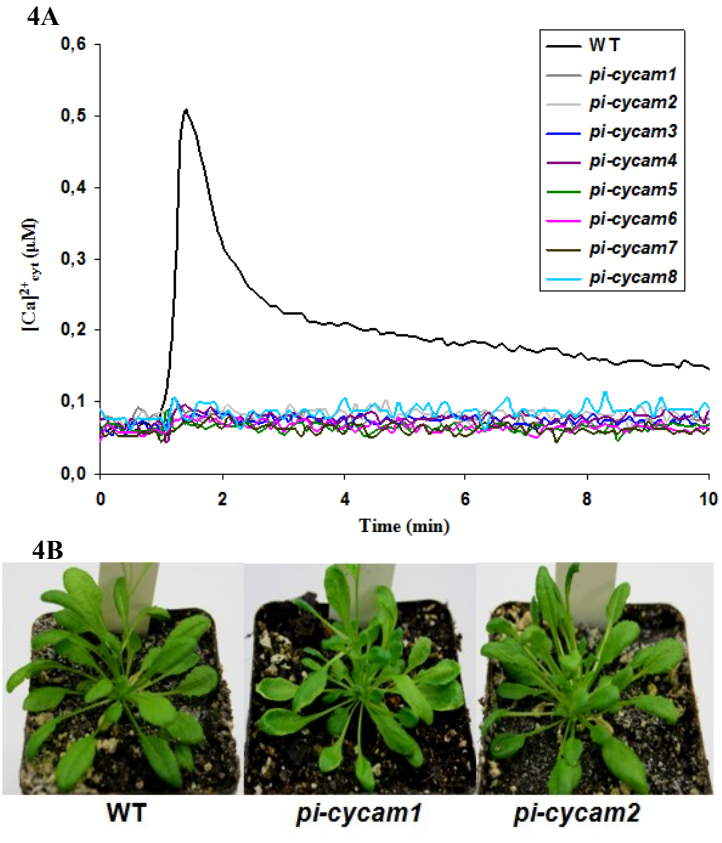


Figure 4. Screening for mutants which do not respond to Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation. M_2 seedlings from the individual M_1 plants were used for the mutant screening. About 70% of roots from the individual M_2 seedlings were challenged with Pi-504. *pi-cycam1* to *pi-cycam8* did not respond to Pi-504 induced $[Ca^{2+}]_{cyt}$ elevation. The WT seedlings expressing cytosolic apoaequorin served as control **(A)**. Three weeks old homozygous mutants grown under short day (8h/16h - light/dark) condition at 22°C with a light intensity of 100 µmol photons m^{-2} s^{-1} **(B)**.

resulted in the complete restoration of $[Ca^{2+}]_{cyt}$ elevation to Pi-504 in ~25% of F_2 progenies. Therefore, *Pi-CYCAM* is a recessive and qualitative trait. Complementation analysis was done by crossing *pi-cycam1* and *pi-cycam2*, and the resulted F_1 progenies did not show $[Ca^{2+}]_{cyt}$ elevation to Pi-504 and thus the mutation is allelic (Supplemental Material 2). Henceforth, the mutants are named as *pi-cycam1-1* and *pi-cycam1-2*.

pi-cycam1 responds to *A. brassicae* toxin preparation

P. indica-colonised plants confer resistance against a wide range of pathogenic fungi including powdery mildew fungi, *Blumeria graminis* f.sp. *hordei* and root rot fungi, *Fusarium culmorum* in barley and *Golovinomyces orontii* in *A. thaliana* (Schäfer et al., 2007; Camehl et. al., 2010; Jacobs et al., 2011). *A. brassicae* is a necrotrophic fungus infecting the members of crucifers including *A. thaliana* and the pathogenicity factor is its host specific toxin. The host specific *A. brassicae*-toxin (Ab-toxin) preparation mimics pathogenic fungus on symptom development and growth inhibition (Moebius and Hertweck, 2009; Pedras and Khallaf, 2012). The response of *pi-cycam1-1* and *pi-cycam1-2* was also tested to *Alternaria brassicae*-toxin preparation for $[Ca^{2+}]_{cyt}$ elevation. Interestingly, both mutants responded to Ab-toxin with regard to the $[Ca^{2+}]_{cyt}$ elevation (Figure 5). However, the $[Ca^{2+}]_{cyt}$ elevation in response to Ab-toxin in both mutants were marginally lower than that of WT.

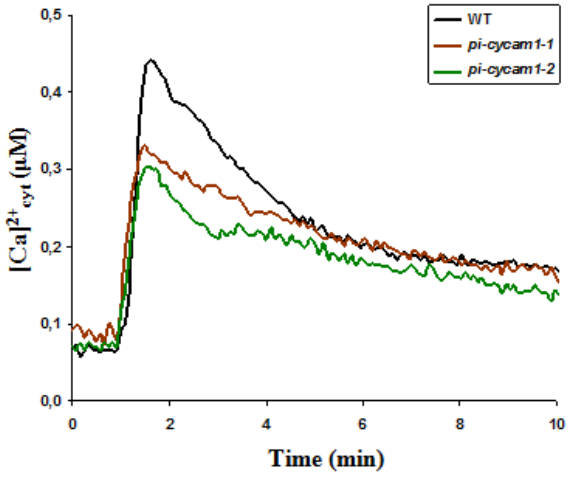


Figure 5. The response of *pi-cycam* to Ab-toxin. The roots of both *pi-cycam1-1* and *pi-cycam1-2* were challenged with 50 µl Ab-toxin preparation. Both mutants responded to the toxin, but to a lesser extend. WT seedlings served as control. All curves represent mean ± SE of 4 independent experiments with 8 replications in each experiment.

Plant cells are often desensitised or lose their capacity to respond to consecutive treatments with the same stimulus called "refractory behavior", but remain sensitive to different stimulus, perceived by another receptor (Blume et al., 2000). To test whether $[Ca^{2+}]_{cyt}$ responses induced by Pi-504 and Ab-toxin have this refractory behavior, roots of *pi-cycam1-2* and WT (pMAQ2) were challenged first and also subsequently with Pi-504 and Ab-toxin

preparation. Pi-504 or *A. brassicae*-toxin was applied to WT seedling roots at the beginning of the experiment as first stimulus and 10 min later when the $[Ca^{2+}]_{cyt}$ induced by the first application is on its descent, a second application of the same stimulus evoked a weak responses in roots ('refractory') (Figure 6A). Similarly, WT roots treated at first with Pi-504 then followed a second application of Ab-toxin induced a strong and significant $[Ca^{2+}]_{cyt}$ elevation in WT roots indicating that the system is still competent (Figure 6A). In *pi-cycam* roots, the first application of Pi-504 followed by the second application of Ab-toxin evoked a transient $[Ca^{2+}]_{cyt}$ elevation (Figure 6B). These results clearly demonstrate that different genes are involved in Pi-504- and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevation. Interestingly, the kinase inhibitor staurosporine treatment completely blocked the $[Ca^{2+}]_{cyt}$ elevation induced by Pi-504 and Ab-toxin in both WT and *Pi-CYCAM* mutants which clearly indicate that the presence of kinase domain(s) in these genes (Figures 6C-D).

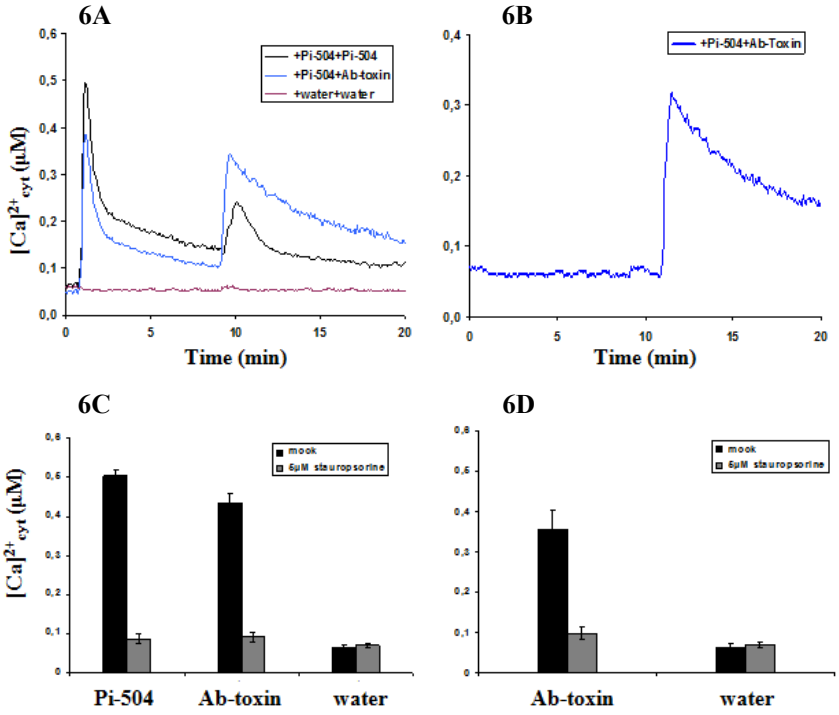


Figure 6. Refractory behaviour of bMAMPs and toxin on the time course of $[Ca^{2+}]_{cyt}$ changes induced in WT and *pi-cycam1-2* seedling roots in the competition assay. Mean (± SE, n=24) of Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation when WT and mutant roots were treated first with Pi-504 and subsequently 10 min later with Ab-toxin preparation **(A)** and **(B)**. Incubation of roots in 5 µM staurosporine completely blocked Pi-504- and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevation in WT **(C)** and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevation in *pi-cycam1-2* **(D)**.

Pi-CYCAM mutants are impaired in growth promotion to Pi-504 and *P. indica*

Pi-504 did not promote the growth of *pi-cycam1-1* and *pi-cycam1-2* unlike WT, when they were grown on MS medium supplemented with Pi-504 (Figures 7A-B). The growth promotion by the fungus was significantly reduced in mutants in terms of seedling biomass compared to WT (Figures 7C-D). The fitness of the seedlings to photosystems were also

determined by measuring various chlorophyll fluorescence parameters e.g. maximum quantum yield of photosystem II (PSII) (F_v/F_m), quantum yield of PSII (Φ_{PSII}), photochemical quenching or proportion of open PS II (qP) and nonphotochemical quenching (NPQ) after a dark adaptation for 20 min using a Fluorocam (Maxwell and Johnson, 2000; Wagner et al., 2008). Dark adapted values of F_v/F_m did not show any difference in *pi-ycam1-1* and *cycam1-2*

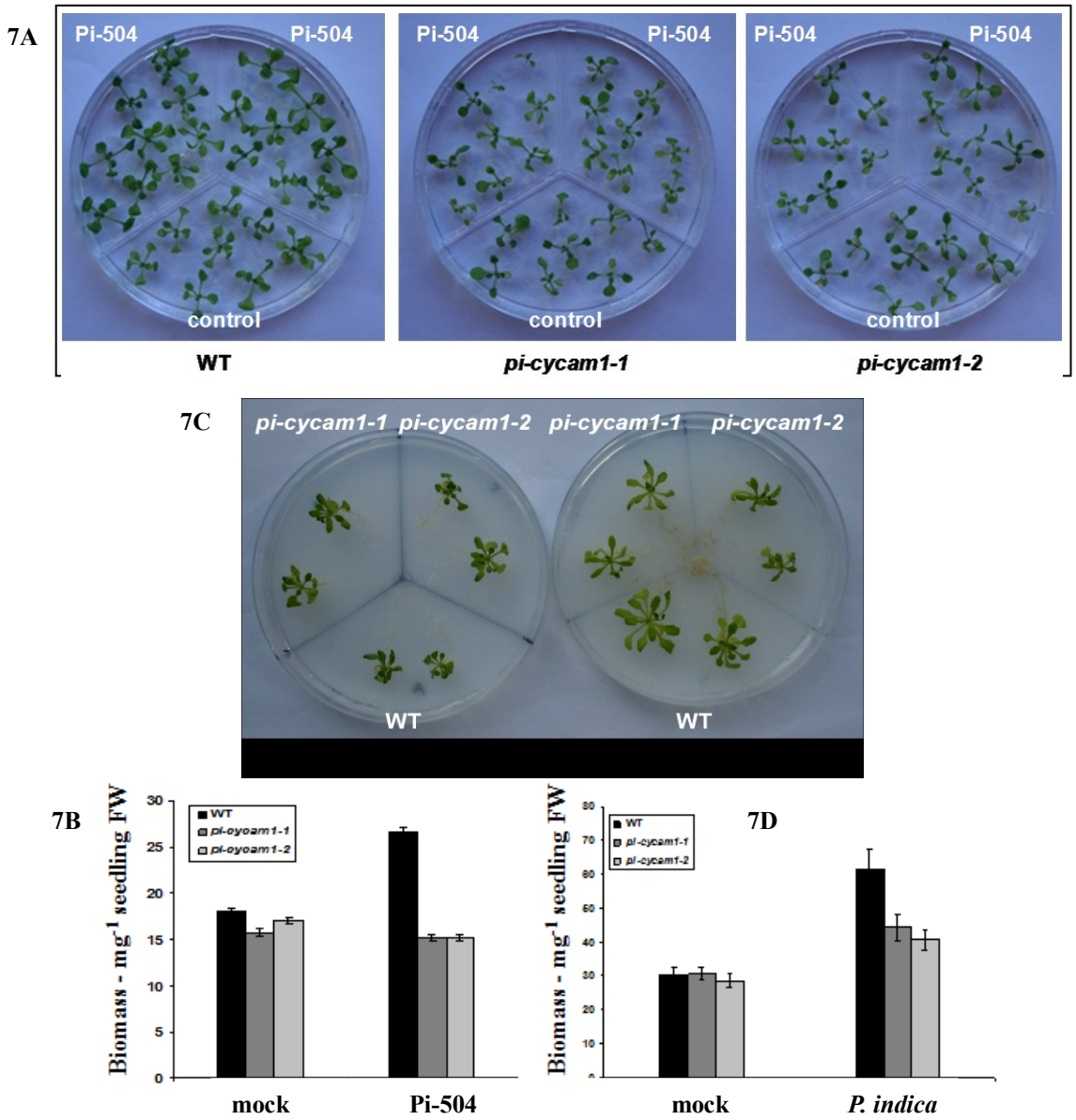
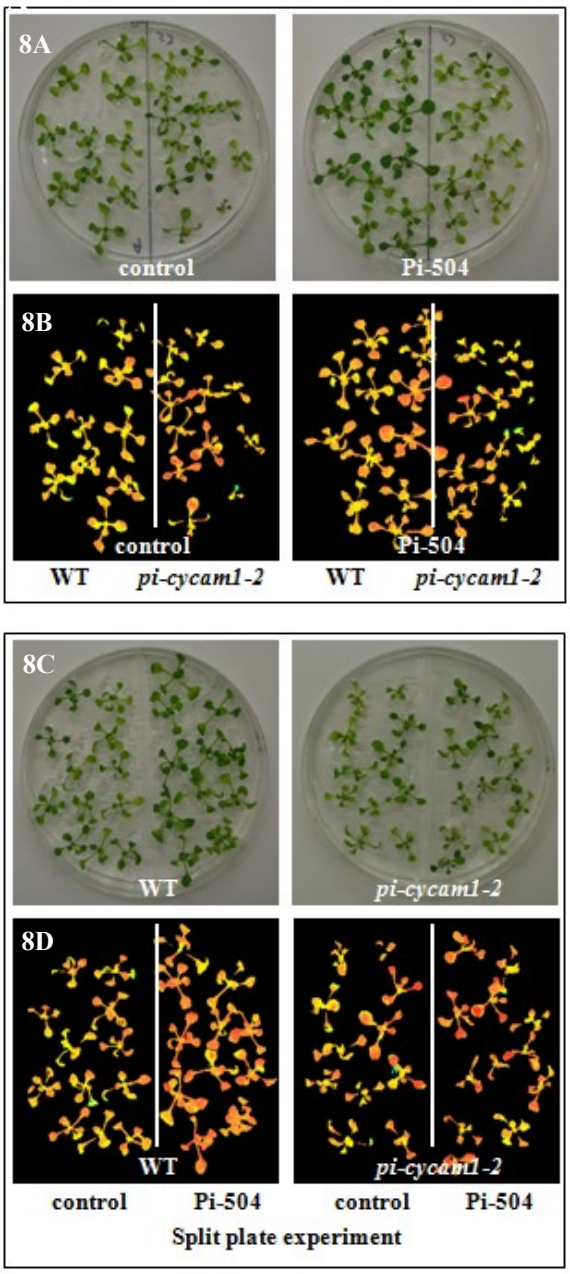


Figure 7. *pi-cycam* showed no growth promotion to Pi-504 and reduced growth promotion to *P. indica*. WT and *pi-cycam* were grown on MS supplemented with 3% of Pi-504 at a strength used to measure $[Ca^{2+}]_{cyt}$ elevation, under LD condition for 14 days. Pi-504 promoted the growth of WT Arabidopsis seedling but not of *pi-cycam1-1* and *pi-cycam1-2* **(A)** and the growth promotion is quantified as biomass on fresh weight (FW) basis **(B)**. Mock treatment was done with sterile water. The growth promotion by *P. indica* was significantly reduced in both mutants compared to WT **(C)** and was quantified as biomass on FW **(D)**. Mock treatment was done with KM plug. The pictures are representative and values are mean ± SE of 4 independent experiments with 50 seedlings for Pi-504 treatment and 24 seedlings for *P. indica* cocultivation in a treatment in each experiment.

compared to WT and is more than 0.83 (Figure 8A-E). However, Φ_{PSII}, NPQ and qP were marginally but significantly decreased in the mutants compared to WT (Figure 8E). Thus, the efficiency of the photosynthetic electron transport (Φ_{PSII}, qP) and the ability of heat dissipation of photochemical energy (NPQ) are impaired in the chloroplasts of *pi-cycam1-1* and *pi-cycam1-2* to a lesser extent. We could demonstrate the direct involvement of *Pi-CYCAM* on the efficiency of electron flow and in photo- and nonphoto-chemical quenching of plastoquinone pool in the chloroplast.



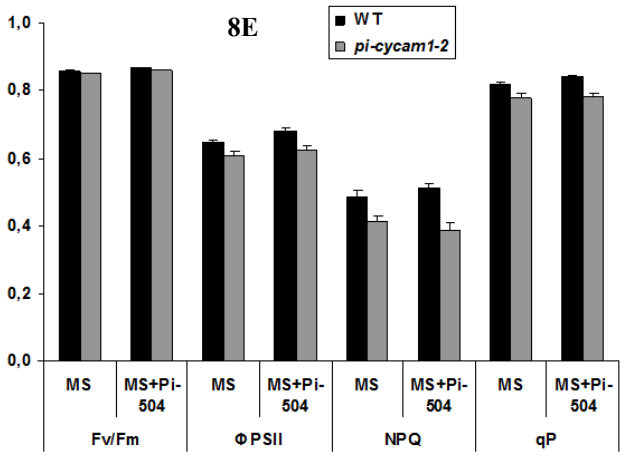


Figure 8. Photosynthetic parameters are affected in *pi-cycam1-1* and *pi-cycam1-2* seedlings. Mutants and WT were grown on MS medium supplemented with Pi-504 as described earlier for 14 days. The maximum quantum yield of PS II (F_v/F_m) is not affected, quantum yield of PSII (Φ_{PSII}), photochemical quenching or proportion of open PS II (qP) and non photochemical quenching (NPQ) are impaired in *pi-cycam* **(A-E)**. The representative pictures were taken **(A)** and also from split plate experiment **(C)**; **(B)** and **(D)** are false images in fluorocam from **(A)** and **(C)** respectively. Pi-504 has no and reduced effect on different photosynthetic parameters in WT and *Pi-CYCAM* mutants respectively **(E)**. The pictures are representative and the values are mean ± SE of 4 independent experiments with 50 seedlings per line per treatment in each experiment.

Loss-of-function of gene involved in Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation impairs the expression of genes involved in primary metabolism

The beneficial interaction of *P. indica* with the host plants is accompanied by a better acquisition of nitrogen (N), phosphorus (P), sulphur (S) and iron (Fe) from the environment by modulating different genes involved in the primary metabolism (Sherameti et al., 2005; Yadav et al., 2010). We have checked the expression of *Nia1* and *Nia2* involved in nitrogen metabolism (Sherametti et al., 2005) and *Pht1.1* and *Pht1.5* which play a critical role in the mobilization of phosphorus to the plant (Yadav et al., 2010) in both *pi-cycam1*-2 and WT seedlings grown on MS medium supplemented with Pi-504 and cocultivated with *P. indica*. The expression levels of *Nia1*, *Nia2*, *Pht1.1* and *Pht1.5* are highly upregulated in WT seedlings grown with Pi-504 and the fungus, whereas these genes were less regulated in *pi-cycam1-2* except for *Nia2* with the fungus (Figure 9).

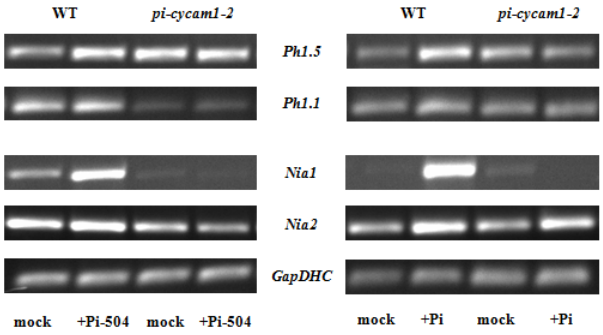


Figure 9. *Pi-CYCAM* mutant modulates the expression of genes involved in primary metabolism. Mutants and WT were grown on MS medium supplemented with Pi-504 for 14 days or cocultivated with *P. indica* for 6 days were harvested for RNA extraction. The upregulation of *Nia1* and *Nia2* involved in nitrogen metabolism and the phosphate transporters *Ph1.1* and *Ph1.5* are shown in WT either treated with Pi-504 or cocultivated with *P. indica*, but not in *pi-cycam1-2*. Mock treatment was done either with water or with KM plug in the respective experiments. The pictures are representative of 4 independent experiments with 3 replications each.

***pi-cycam1* seedlings are sensitive to abiotic and biotic stress**

The sensitivity of *pi-cycam* to NaCl and H_2O_2 were analysed by growing the mutants and WT on MS medium amended with 100 mM NaCl and 10 mM H_2O_2. After 3 weeks of growth, both treatments significantly reduced the biomass in mutants and WT but the extent of reduction was more in *pi-cycam1-1* and *pi-cycam1-2* (Figure 10A). This result suggests that *Pi- CYCAM1* is involved in salt and oxidative stress. Further, the mutants were extremely susceptible to *A. brassicae* infection and its toxin preparation as evidenced by high incidence of disease and high reduction of biomass compared to WT (Figures 10B-E) which clearly demonstrates that there is a break down of both defense and growth in mutants. The result of toxin influence on both *pi-cycam1* and WT prompted us to compare these lines with flg22

(flagellin22), a well-known bacterial PAMP which inhibit the growth of Arabidopsis seedlings (Anderson et al., 2011; Ranf et al., 2012). flg22 treatment significantly inhibited the growth but did not show any preferential effect on the mutants and WT seedlings (Figures 10D-E). Moreover, both mutants responded normally to flg22 like WT in terms $[Ca^{2+}]_{cyt}$ elevation when challenged with 10-day old cotyledons (data not shown).

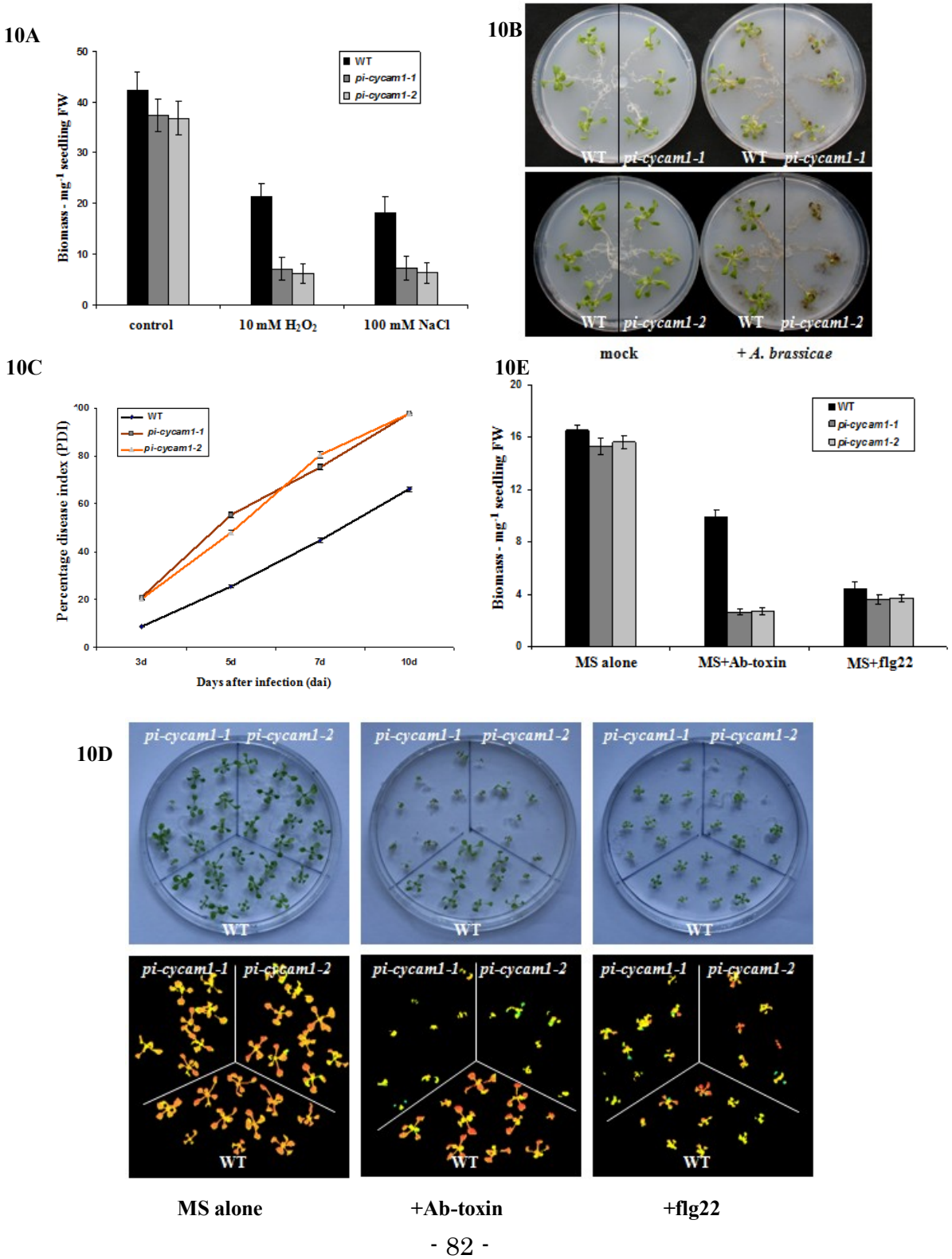


Figure 10. *pi-cycam1* seedlings are more sensitive to abiotic and biotic stress. Mutant and WT seedlings were grown on MS medium supplemented with 10 mM H_2O_2 or 100 mM NaCl for 20 days **(A)**. The values are mean ± SE of four independent experiments with more than 50 seedlings of each line in one treatment per experiment. **(B)** and **(C)** show both mutants are highly susceptible to *A. brassicae* infection compared to WT. The percentage disease index (PDI) were determined at 3, 5, 7 and 10 days after inoculation (dai) of seedling leaves of different lines with spore suspension. The representative pictures were taken at 5 dai and the values are mean ± SE of 4 independent experiments with 24 seedlings of each line in a treatment in each experiment. The response of *pi-cycam1* seedlings to Ab-toxin and flg22 in MS medium supplemented with 3% of Ab-toxin at a strength used to measure $[Ca^{2+}]_{cyt}$ elevation and 1 µM flg22, under LD condition for 14 days **(D)** and **(E)**. The growth inhibition was determined by taking biomass on FW basis. The pictures are representative and values are mean ± SE of four independent experiments with more than 50 seedlings per line per treatment in each experiment.

A. brassicae induce more H_2O_2 and ROS production in *pi-cycam1* seedlings

Phytopathogens- and their PAMPs-induced ROS and H_2O_2 production in treated plant cells (Lecourieux et. al., 2006; Peleg-Grossman et al., 2012) whereas, bMAMPs from AMF and *P. indica* did not induce H_2O_2 production (Navazio et al., 2007; Vadassery et al., 2009). ROS and H_2O_2 levels were measured from the roots and shoots of WT and mutants seedlings after treating them with Ab-toxin and inoculated with *A. brassicae* spores. Ab-toxin significantly induced ROS and H_2O_2 in both WT and *pi-cycam1* compared to the mock treatment; but the induction was much higher in mutants compared to WT (Figures 11A-B). Interestingly, Pi-504 treatment did not induce ROS and H_2O_2 in both WT and mutants (Figures 11A-B). Three days after infection (dai) of *A. brassicae*, we found an enhanced level of H_2O_2 and ROS in roots and shoots of the mutants and WT, but the proportion of increase was much higher in mutants (Figures 11C-D). We also studied the expression of different ROS and H_2O_2 marker genes in the *A. brassicae* infected seedlings. The mRNA levels of redox regulated transcription factor1 (*RRTF1*), basic helix-loop-helix (*bHLH*) TF, zinc finger (*ZF*) CCCH, apetala2/ethylene responsive element binding protein (*AP2/EREBP*), disease and stress related protein (*DSR*) and o-methyltransferase (*OMT*); ROS marker genes, and oxidative signal-inducible1 (*OXI1*) and dark inducible11 (DIN11); H_2O_2 marker genes (Rentel et al., 2004; Khandelwal et al., 2008; Mehterov et al., 2012), were highly upregulated in mutants compared to WT (Supplemental Material 3). Furthermore, the enzymes involved in ROS scavenging, such as the Fe-superoxide dismutase1 (FSD1), Cu-superoxide dismutase2 (CSD2), ascorbate peroxidase1 (APX1), monodehydroascorbate reductase2 (MDAR2) and dihydroascorbate reductase5 (DHAR5) were downregulated in shoots of *pi-cycam1* (Supplemental Material 3). Both the elevated H_2O_2/ROS levels and low ROS scavenging enzymes in mutants are responsible for their higher susceptibility to *A. brassicae* infection. We further tested the

sensitivity of mutants to oxidative stress induced by the catalase inhibitor, 3-aminotriazole (3AT) and the herbicide paraquat (PQ), agents that cause the endogenous accumulation of hydrogen peroxide and superoxide radicals respectively (Mehterov et al., 2012). Interestingly, both mutants suffered more when they were grown on MS medium amended with 3AT and PQ and the degree of susceptibility was more with PQ which further support our finding that the *A. brassicae*-induced burst of ROS in *pi-cycam1-1* and *pi-cycam1-2* makes the cells more toxic and weak, thereby highly susceptible to the infection (Supplemental Material 4).

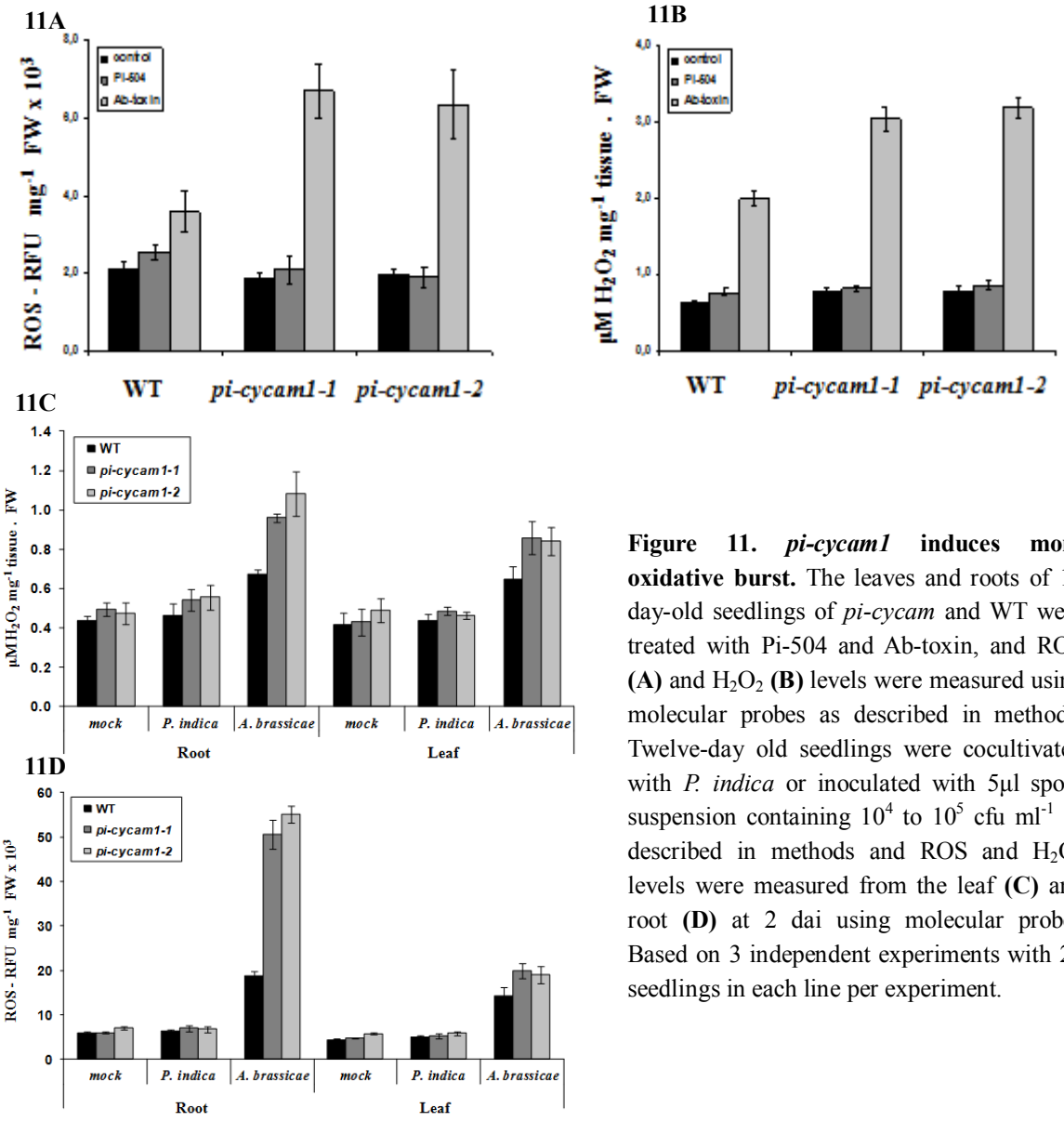


Figure 11. *pi-cycam1* induces more oxidative burst. The leaves and roots of 14 day-old seedlings of *pi-cycam* and WT were treated with Pi-504 and Ab-toxin, and ROS **(A)** and H_2O_2 **(B)** levels were measured using molecular probes as described in methods. Twelve-day old seedlings were cocultivated with *P. indica* or inoculated with 5µl spore suspension containing 10^4 to 10^5 cfu ml^{-1} as described in methods and ROS and H_2O_2 levels were measured from the leaf **(C)** and root **(D)** at 2 dai using molecular probes Based on 3 independent experiments with 24 seedlings in each line per experiment.

Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation is upstream of RRTF1-induced ROS amplification

$[Ca^{2+}]_{cyt}$ and ROS signaling are the earliest events which induce plant defense responses during pathogen invasion or on treatment with PAMPs (Zhang et al., 2009; Torres, 2010;

Heller and Tudzynski, 2011; Takahashi et al., 2011). $[Ca^{2+}]_{cyt}$ elevations have been reported both upstream and downstream of ROS production indicating complex spatiotemporal Ca^{2+} and ROS elevation mechanisms (Kawano and Muto, 2000; Blume et al., 2000). As *RRTF1* acts as a hub to amplify ROS production in response to *A. brassicae* infection and is upstream of oxidative burst (Kerchev et al., 2013; Matsuo et al., 2013 revision), we used *RRTF1* mutant with reduced ROS level and its overexpressors (*oe18* and *oe20*) which accumulate more ROS to the cytotoxic level (Matsuo et al., 2013 revision) to elucidate whether Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation is upstream of ROS induction by *A. brassicae* or its toxin. We crossed *rrtf1, oe18* and *oe20* with WT aequorin (pMAQ2). Interestingly, toxin-induced $[Ca^{2+}]_{cyt}$ elevation did not change significantly in the F_3 progenies of *rrtf1*xPMAQ2 and *oe20*xPMAQ2 crossed lines (Figure 12A) which clearly demonstrate that *A. brassicae*-toxin-induced $[Ca^{2+}]_{cyt}$ elevation is upstream of ROS amplification by *RRTF1*. Pi-504-induced $[Ca^{2+}]_{cyt}$ elevations are also not altered in the F_3 progenies of crossed lines (Figure 12A). We extended our study with different ROS inhibitors e.g. diphenyleneiodonium (DPI), VAS2870, rotenone and N-acetly-L-cysteine (NAC); and ROS enhancers e.g. paraquat and 3-AT; which also did not affect the $[Ca^{2+}]_{cyt}$ elevation induced by Pi-504, and *A. brassicae*-toxin in WT plants (Figure 12B) which further reinstate that the $[Ca^{2+}]_{cyt}$ elevations induced by Pi-504, and *A. brassicae*-toxin are upstream of ROS induction.

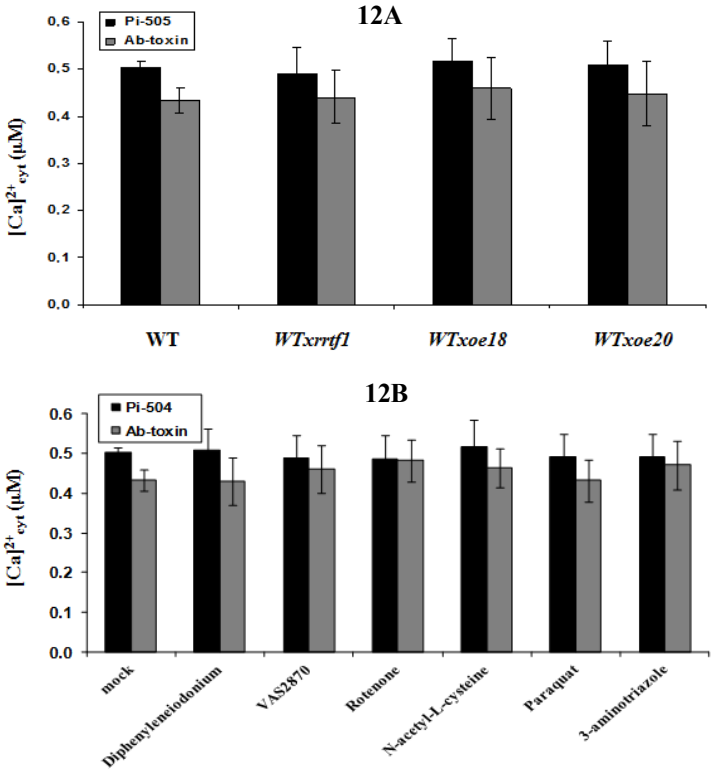


Figure 12. Pi-504- and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevation are upstream of ROS amplification in Arabidopsis roots. Roots of 16-18-day old F_3 seedlings of different crossed lines *WTxrrtf1*, *WTxoe18* and *WTxoe20* were prepared as described in Methods, and treated with 50µl of Pi-504 and Ab-toxin preparation. The crossed lines did not show significant changes in the $[Ca^{2+}]_{cyt}$ elevation in response to different stimuli tested compared to WT control **(A)**. The roots of 18-day old WT seedlings were incubated in different ROS inhibitors and enhancers for 1 h, then challenged with 50µl of Pi-504 and Ab-toxin preparation. Different ROS inhibitors or enhancers did not change the $[Ca^{2+}]_{cyt}$ levels induced by Pi-504 and Ab-toxin **(B)**. Water served as control and gave background readings. All values represent mean peak ± SE of three independent experiments with eight replications in each experiment.

DISCUSSION

Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation promotes growth in Arabidopsis

Perception of signals from both beneficial fungi or/and its bMAMPs and pathogenic fungi or/and its PAMPs transiently increase $[Ca^{2+}]_{cyt}$ in their host cells is an essential signaling event during the very early phase of plant-microbe interactions, modulating an array of growth and defense processes essential for the survival of plants. (Blume et al., 2000; Lecourieux et al., 2006; Navazio et al., 2007; Vadassery et al., 2009; Ranf et al., 2012). Similarly, in different pathogenic interactions, PAMP-induced $[Ca^{2+}]_{cyt}$ elevations have been reported for pep25 from *Phytophthora sojae*, flg22 from flagellated bacterium and elf18 from elongation factor Tu in *Arabidopsis* seedlings and inhibited the growth of Arabidopsis seedlings (Hu et al., 2009; Jeworutzki et al., 2010; Ranf et al., 2012). A trisaccharide from the growth promoting CWE of the beneficial root endophyte *P. indica* induced $[Ca^{2+}]_{cyt}$ elevation in a dose dependent manner with distinct Ca^{2+} signature in their lag time, peak time, duration and shape (Figure 2A-B). The purified trisaccharide has a molecular mass of *m/z* 505.1748 (Figure 1A-B). Interestingly Pi-504 promoted the growth of Arabidopsis and tobacco as the fungus does (Figures 3A-F). This result shows that growth promotion can be uncoupled from the colonization of the roots by the fungus although Pi-504 cannot completely mimic growth promotion induced by the fungus. The symbiotic interaction of *P. indica* with plant roots results in acquisition of more nitrogen by modulating N_2 metabolism and phosphates uptake from the environment (Sherameti et al., 2005; Yadav et al., 2010). In the present study, Pi-504 induces $[Ca^{2+}]_{cyt}$ elevations and also promotes growth in Arabidopsis by positively modulating the genes involved in N_2 metabolism and phosphates uptake (Figure 9).

pi-cycam1-1 and *pi-cycam1-2* do not induce $[Ca^{2+}]_{cyt}$ elevations and are impaired in growth promotion

Large scale screening of an EMS-mutagenized pMAQ2 M2 seedlings resulted in the isolation of *Pi-CYCAM* mutants which do not induce $[Ca^{2+}]_{cyt}$ elevation in response to Pi-504 (Figure 4A and Supplemental Material 1). Interestingly, Pi-504 which does not induce $[Ca^{2+}]_{cyt}$ elevation also fails to promote growth in mutants (Figures 7A-B). The response of *Pi-CYCAM* mutants to *P. indica* shows that the growth promotion is very much reduced (>70%) in both mutants compared to WT (Figures 7C-D). The growth promotion in *Arabidopsis* and tobacco seedlings by the fungus is attributed to the enhanced nitrate uptake and expression of *Nia2* in

roots and shoots (Sherameti et al., 2005). Uptake and transport of phosphorus is also stimulated by the fungus in the colonized roots of maize (Yadav et al., 2010). Recently a phosphate transporter, PiPT from *P. indica* has been purified and crystalised (Pedersen et al., 2013). The expression studies of genes involved in nitrogen metabolism and phosphorus uptake shows that both Pi-504 and the fungus induce higher expression levels of *Nia1* and *Nia2* involved in nitrogen metabolism and *Pht1.1* and *Pht1.5* involved in phosphate uptake are upregulated in WT compared to *pi-cycam1* (Figure 9). Moreover, different photosynthetic parameters, for example, Φ_{PSII}, qP and NPQ are marginally but significantly higher in WT compared to mutants (Figures 8A-E). Thus, the efficiency of the photosynthetic electron transport (Φ_{PSII}, qP) and the ability of heat dissipation of photochemical energy (NPQ) are impaired in the chloroplasts of *pi-cycam1*. Therefore, we propose that the Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation is directly involved in enhanced photosynthesis, nitrogen metabolism and phosphate uptake which in turn leads to growth promotion in Arabidopsis.

Loss of function of *Pi-CYCAM* makes the mutants more sensitive to salt and oxidative stress and also hypersusceptible to *A. brassicae* infection

$[Ca^{2+}]_{cyt}$ is very crucial for the rapid response of plants to biotic and abiotic stress (Lecoureux et al., 2006; Kudla et al., 2010). In the present study, the mutants were more sensitive to salt and oxidative stress (Figure 10A). The crucial role of $[Ca^{2+}]_{cyt}$ has been well demonstrated in the transduction of salt- (Knight et al., 1997; Klüsener et al., 2002) and oxidative- (Klüsener et al. 2002) stress signals in Arabidopsis. Moreover, mutants were also highly sensitive to paraquat and 3-aminotriazole which induces endogenous ROS and H_2O_2 levels respectively in plant cells (Supplemental Material 4). Therefore, the loss-of-function of *Pi-CYCAM1* compromises the adaptation of mutants to salt and oxidative stress conditions. *Pi-CYCAM1* mutants are also highly susceptible to *A. brassicae* infection and more sensitive to *A. brassicae*-toxin (Figures 10B-E). The host specific Ab-toxin preparation behaves exactly like the fungus as it inhibits the growth of seedlings in terms of biomass. High PDI and low biomass in both mutants (Figures 10C and 10E) clearly demonstrate that the *Pi-CYCAM* is very crucial for conferring resistance against pathogenic fungus. A relatively high tolerance of WT to *A. brassicae* infection and to Ab-toxin further reinstate that the $[Ca^{2+}]_{cyt}$ elevations induced by Pi-504 is very much essential for the better adaptation of plants to biotic and abiotic stress. Moreover, Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation may be repressing the signaling cascades of Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevation involved in enhanced host susceptibility to

the pathogens (effector triggered susceptibility). We propose that Pi-504-induced $[Ca^{2+}]_{cyt}$ signaling cascade is evolutionarily conserved to have better adaptation to biotic and biotic stress in plants. The differential response of WT and *pi-cycam1* to Ab-toxin is not seen when they were grown with flg22 (Figures 10D-E) even though flg22 could induce $[Ca^{2+}]_{cyt}$ elevation in both WT and *pi-cycam1* (data not shown). Therefore the differential response of WT and *pi-cycam1* to abiotic and biotic stress is attributed to the loss-of-function of $[Ca^{2+}]_{cyt}$ elevations induced by Pi-504. Interestingly, 'refractory' studies and the total inhibition of $[Ca^{2+}]_{cyt}$ elevation by saturosporine (Figures 6A-D) clearly demonstrate that Pi-504- and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ genes have functional kinase domain(s). The total inhibition of $[Ca^{2+}]_{cyt}$ elevation by staurosporine has been shown independently in cryptogein-tobacco suspension culture (Lecourieux et al., 2002), Myc factor-soybean suspension culture (Navazio et al., 2007), CWE of *P. indica*-Arabidopsis root (Vadassery et al., 2009) and ergosterol-tobacco systems (Vatsa et al., 2011).

Hypersusceptibility of *pi-cycam1* to *A. brassicae* is positively correlated to ROS amplification

Ab-toxin treatment and seedling infection with the spores of *A. brassicae* induced high ROS/H_2O_2 levels in roots and leaves of WT and *pi-cycam1*, but the induction was much higher in both mutants (Figures 11A-D) which are highly susceptible to the fungus and sensitive to the toxin (Figures 10B-E). ROS is important for the recognition of pathogens and activation of basal defense reactions (c.f. Torres, 2010; Heller and Tudzynski, 2011). Necrotrophic fungi e.g. *A. brassisicola, A. alternata* f. sp. *lycopersici* and *B. cinerea* use these basal defense for the successive colonization in their hosts by producing effectors/toxins (Gechev et al., 2004; Govrin et al., 2006; Lenz et al., 2011 and Su'udi et al., 2011; Zhao et al., 2013). Higher levels of H_2O_2/ROS production in *pi-cycam1* is correlated to the loss of function of $[Ca^{2+}]_{cyt}$ elevation induced by Pi-504 (Figures 11A-D). The toxin-induced Ca^{2+} signaling cascade also induced much higher level of ROS/H_2O_2 in mutants compared to WT. Therefore, Pi-504-induced Ca^{2+} signaling cascade represses Ab-toxin-induced Ca^{2+} signaling cascade thereby reduces the higher level of ROS burst to a level at which ROS act as a signaling molecule rather than a phytotoxic compound.

ROS with signaling functions include H_2O_2, singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$) and superoxide anion radical ($O_2^{\cdot-}$) (Laloi et al., 2004; Mehterov et al., 2012). We have checked the expression levels of ROS marker genes which are preferentially regulated by

H_2O_2, $O_2^{\cdot-}$ and common ROS. Interestingly, all these ROS marker genes are strongly upregulated in both WT and *pi-cycam1* seedlings infected with *A. brassicae* (Supplemental Material 3). However the induction of ROS marker genes were much higher in mutants which accumulated high level of ROS/H_2O_2 in *pi-cycam1*, and are hypersusceptible to *A. brassicae* infection and sensitive to its toxin (Figures 10B-E and 11A-D). Among the common ROS marker genes, *RRTF1*, *bHLH*, *JRG1*, *AP2/EREBP* TF and *DSR* are induced by abiotic and biotic stress in Arabidopsis (Toufighi et al., 2005; Khandelval et al., 2008; Mehterov et al., 2012). OXI1 with serine/threonine kinase activity and protein phosphorelation is induced by H_2O_2, and is necessary for oxidative burst-mediated signaling to activate defense responses against pathogen infections and PAMP treatments in Arabidopsis (Rentel et al., 2004). However, the beneficial fungus *P. indica* repressed *RRTF1* and *OXI1*, and thereby ROS/H_2O_2 production in the colonized roots to promote the plant growth but did not activate defense responses (Camehl et al., 2011; Matsuo et al., 2013 revision). Another striking observation is that *A. brassicae* infection activates ROS accumulation by repressing ROS scavenging genes particularly in the mutants which are more susceptible to *A. brassicae* (Figures 11A-B and Supplemental Material 3). Interestingly, *A. brassicae* infection in WT induced higher levels of ROS scavenging genes which are localised in chloroplast (*FSD1*, *CSD2* and *DHAR5*; TAIR) and cytoplasm (*APX1*, *MDAR2* and *DHAR5*; TAIR) compared to *pi-cycam1* (Supplemental Material 3). Therefore, it is postulated that Pi-504-induced $[Ca^{2+}]_{cyt}$ is also involved in the activation of ROS scavenging genes which further reduces ROS induced by Ab-toxin and the fungus. This is very clear with the low level of ROS in WT treated with toxin or inoculated with *A. brassicae* (Figures 11A-D and Supplemental Material 3). Antioxidant enzymes e.g. superoxide dismutases, reductases and peroxidases together with antioxidants such as ascorbate and glutathione detoxify the ROS and stabilize the redox state in the different cellular compartments (Alscher et al., 2002; Asada, 2006). Taken together, loss of function of Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation together with toxin-induced $[Ca^{2+}]_{cyt}$ elevation amplifies ROS/H_2O_2 generation by activating different ROS genes and repressing ROS scavenging enzymes to accumulate ROS to a harmful level which predisposes *pi-cycam1* to higher level of *A. brassicae* infection and sensitivity to its toxin.

$[Ca^{2+}]_{cyt}$ elevation induced by Pi-504 is upstream of RRTF1-mediated ROS amplification

Ca^{2+} fluxes and protein phosphorylation are required for the controlled generation of ROS (Takahashi et al., 2011) and ROS thus generated in turn activates membrane bound Ca^{2+}

channels (Mori and Schroeder, 2004). Among the 10 Rboh (respiratory burst oxidase homolog)-NADPH oxidase genes in Arabidopsis, RbohD and RbohF are involved in defense responses (Marino et al., 2012). RbohD and RbohF are synergistically activated by phosphorylation and binding of Ca^{2+} to their hydrophilic N-terminal regions (Ogasawara et al., 2008). As *RRTF1* acts as a hub to amplify ROS production in response to *A. brassicae* infection, and is upstream of oxidative burst (Mehterov et al., 2012; Kerchev et al., 2013; Matsuo et al., 2013 revision), we used *RRTF1* mutant and its overexpressors (oe) to elucidate whether Pi-504- and Ab-toxin- induced $[Ca^{2+}]_{cyt}$ elevation is upstream of ROS induction by *A. brassicae* or its toxin. Both genetic and pharmacological studies demonstrate that Pi-504- and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevation did not change significantly in the F_3 progenies of crossed lines (Figures 12A-B) which clearly demonstrate that Pi-504- and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevation is upstream of *RRTF1*.

In conclusion, we could show that a trisaccharide (*m/z* 505.1748) from *P. indica*-CWE promotes growth of *A. thaliana* and tobacco as the fungus does, induces a $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis root and confers resistance/tolerance to abiotic and biotic stress. The loss-of-function of *Pi-CYCAM* results in no/reduced growth promotion to Pi-504 and *P. indica*, increased susceptibility to *A. brassicae* infection and sensitivity salt and oxidative stress. Both Ab-toxin treatment and *A. brassicae* infection results in ROS burst in *Pi-CYCAM* mutants to a phytotoxic level which further predispose the mutants to higher sensitivity to abiotic stress and hypersusceptibility to *A. brassicae* infection, whereas Pi-504-induced Ca^{2+} signaling represses the ROS burst by downregulating the ROS marker genes and upregulating ROS scavenging enzymes. Hence, Pi-504 induced $[Ca^{2+}]_{cyt}$ elevation positively regulates the growth, and modulates defense in Arabidopsis.

METHODS

Plant material and growth

Transgenic *A. thaliana* expressing cytosolic apoaequorin (Aeq^{cyt}) in Col-0 background (pMAQ2) was a kind gift from Marc Knight (Knight et al., 1991). Mutagenesis was performed using 0.2 and 0.4% ethane methyl sulfonate as per the standard protocol (Arabidopsis: A Laboratory Manual, ISBN: 0–87969–573–0). M_1 plants were harvested individually and mutation rate was estimated to be 1-2% based on albino or 'chlorophyll-sectoring' analysis (Ranf et al., 2012). Individual M_2 seeds were grown on Hoagland (HL) medium containing 1% agar in square plates (120X120X16mm). After stratification at 4°C for

48 h, plates were kept vertically to grow the roots on the surface of the medium and incubated for 16-18 days at 20±1°C under long day (LD) condition with light intensity 80 µmol m^{-2} sec^{-1} (Vadassery et al., 2009; Johnson et al., 2011a).

Culturing and growth conditions of *P. indica* and *A. brassicae*

P. indica was cultured and maintained on Kaefer medium (KM), pH 6.5 as described by Johnson et al. (2011a). The plates were inoculated with 5 mm diameter fungal plugs and were kept at 22°C in the dark for 3 to 4 weeks. The fungus was also grown on KM broth for 18 days at temperature 22°C in complete darkness on a horizontal rotating shaker at 50 rpm for preparing the cell wall extract (Vadassery et al., 2009; Johnson et al., 2011a). The colonizing efficiency of the fungus was maintained by the periodical cocultivation in Arabidopsis seedlings and re-isolation of the fungus from the internally colonised roots (Johnson et al., 2011b). The pure culture of *A. brassicae* (FSU-3951) was obtained from Jena Microbial Resource Centre, FSU Jena, Germany and the fungus was grown on potato dextrose agar (PDA) medium (pH 6.5-6.7) at 20±1°C in a temperature controlled chamber under 12/12 h light/dark and 75% relative humidity for 2 weeks. To maintain the virulence of *A. brassicae*, the fungus was inoculated to Arabidopsis seedlings and re-isolated from the infected tissues periodically.

Purification of Pi-504 from Pi-CWE

The CWE was prepared as per the protocol of Anderson-Prouty and Albersheim (1975) with modifications (Vadassery et al., 2009; Johnson et al., 2011a). Pi-CWE active fractions from LC-18 SPE cartridge were further concentrated in speed vac, precipitated to 80% methanol and the supernatant was collected after centrifuging at 6000g for 5min. The supernatant was evaporated in speed vac, resuspended in ddH_2O and further separated in Roti-Spin Mini having molecular weight cut off with 3 kD (Roth, Germany). The flow through (< 3 kD) was further concentrated in speed vac and dissolved in ddH_2O. Further purification was done by performing HPLC with LC-18-DB column, 25cm x 4.60 mm ID (Suppelco) and then with HPLC Asahipak NH_2P-50 4E column, 25cm x 4.6mmID (Schodex). The active fractions were collected by testing $[Ca^{2+}]_{cyt}$ elevation. The highly active fractions were further pooled, concentrated and resuspended in ddH_2O. Again the active fractions were further separated using UPLC columns first with Acclaim C18 Column, 250 x 2.1 mm, 2.2 µm, (Dionex) and finally with C18 phenyl column, 150x 2.1 mm, 1.7 µm (Phenomenex). In all HPLC and

UPLC separation, acetonitrile:water gradient was used as the mobile phase for 30 min run with a flow rate of 1 ml/min for HPLC and 0,3 ml/min for UPLC columns.

Acid hydrolysis of Pi-504

The lyophilised Pi-504 was treated with 1 N H_2SO_4 in a water bath at 100^0C for 1 h and normalised with 1 N NaOH after cooling and tested for $[Ca^{2+}]_{cyt}$ measurements.

***A. brassicae* toxin preparation**

The toxin from *A. brassicae* culture filtrate was extracted as described earlier (Vidhyasekaran et al., 1997). The crude toxin was further purified by passing it through a Sephadex G100 column and the active fractions were collected, concentrated and lyophilised. The lyophilised powder was resuspended in sterile H_2O and further purified by passing it through a reversed phase Supelclean LC-18 SPE cartridge. The active fractions were collected and used as stimulus for $[Ca^{2+}]_{cyt}$ measurement and growth assays. All active components were lyophilized and resuspended either in sterile H_2O or in respective buffers to perform different experiments.

Mutant screening by $[Ca^{2+}]_{cyt}$ measurement

Aequorin based luminescence measurements were done using 16 to 18-day old individual M_2 plants grown in HL medium (Vadassery et al., 2009; Johnson et al., 2011b). The transgenic Arabidopsis expressing cytosolic apoaequorin in Col-0 background (pMAQ2) served as control (Knight et al., 1991, 1997). Based on the response of seedlings to Pi-504, the putative M_2 mutants were rescued and transferred to pots containing garden soil and vermiculite at 9:1 (v/v) for further screening and validation. The mutant seedlings were grown in a temperature-controlled growth chamber at 20°C under short day (SD) condition (8 h light and 16 h darkness) for 4 weeks followed by LD condition (16 h light and 8 h darkness) with a light intensity of 100 µmol m^{-2} sec^{-1} after keeping tripod and aracon tubes to maintain the purity of the seeds. The seeds were harvested from individual plants and again screened as described earlier to get the homozygote mutants.

Cocultivation of *P. indica* with Arabidopsis seedlings

The cocultivation experiment with *P. indica* was done as described in Johnson et al. (2011b, Method 2). Control seedlings were transfered to plates inoculated with a plaque without fungal hyphae. The plates were sealed and incubated under LD conditions with a light

intensity of 80 µmol m^{-2} sec^{-1}.

Preparation of *A. brassicae* spore suspension and inoculation to seedlings

A. brassicae sporulates heavily in Potato Dextrose Broth (PDB; pH 6.5-6.7). Two-week old fungal plug (5mm diameter) was inoculated to PDB and incubated for 2 weeks as described earlier to sporulate heavily. To harvest the spores, the medium was drained out by filtering through 4 layers of sterilized nylon membrane and washed the hyphae and spores 3 times with sterile H_2O to remove the excess medium. The spores and hyphae were gently homogenized after adding 50 ml of sterile H_2O and filtered through four layers of sterilized nylon membrane to remove the mycelia and hyphae. The spore count was adjusted to 10^4-10^5 colony forming units (cfu) ml^{-1} by serial dilution or a haemocytometer for further studies. For uniform dispersion of spores, 1-2 drops of Tween-20 was added to 100 ml of spore suspension. For seedling infection, after 48 h of transferring the 12-d old seedlings to PNM plates (for details see Johnson et al., 2011b; c1: method2), 6 leaves in the middle whorl per seedling were inoculated with 5 µl of spore suspension containing 10^4-10^5 cfu ml^{-1}. Mock treatment was done with sterile H_2O. The plates were sealed and incubated under LD conditions as described earlier. The progression of disease development determined as percentage disease index (PDI) at 3, 5, 7 and 10 days after inoculation (dai) of spores based on the number and area of leaves infected using standard disease intensity grades.

Abiotic and biotic stress assays

The surface-sterilized seeds of wild type (pMAQ2) and different homozygous $[Ca^{2+}]_{cyt}$ mutants were placed on MS medium containing 0.44% MS salt, 1.3% sucrose, 0.05% MES and 0.8% agar (pH 5.8 with 5 N KOH). Salt and oxidative stress treatments were performed by adding NaCl and H_2O_2 respectively to the MS medium after autoclaving and cooling. WT and *pi-cycam1* were also grown on MS medium alone or supplemented with Pi-504 or Ab-toxin preparation, or different chemicals to the final concentration mentioned in the result section to study their effect. After cold treatment at 4°C for 48 h, plates were incubated at 20°C under LD condition with a light intensity of 80 µmol m^{-2} sec^{-1} for 10-20 days depending on the experiments.

Measurement of photosynthesis parameter

Two-week old *Arabidopsis* seedlings grown on MS medium were dark-adapted at least for 20 min and then the chlorophyll (chl) fluorescence was measured at room temperature using a

video imaging with a pulse amplitude-modulated FluorCam 700F (Photon System Instruments, Czech Republic). Program parameters of FluorCam were essentially according to Wagner et al. (2008). Photosynthesis parameters; quantum yield of PSII (Φ_{PSII}), maximum quantum yield of PSII (Fv/Fm), photochemical quenching (qP) and nonphotochemical quenching (NPQ) were calculated based on Maxwell and Johnson (2000). False color images of the seedlings in plates were obtained as described by Wagner et al. (2008). Chl fluorescence images representing Fs/Fm values are shown, whereas blue represent low F_s/F_m values above a threshold of 0.06 and red represents high F_s/F_m values with an upper threshold limit of 0.17.

Quantitative intracellular H_2O_2 and ROS measurements

Quantitative H_2O_2 measurement from leaves and roots were performed using the Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular probes, Invitrogen) according to the manufacturer's instructions (http://tools.invitrogen.com/content/sfs/-manuals/ mp22188.pdf). Leaf bits of 0.5-1 mm width and root bits of 2-3 cm long were incubated in the reaction mixture for 10 min in dark at room temperature. The fluorescence intensity was quantified with a fluorescence microplate reader (TECAN Infinite 200 plate reader; Crailsheim, Germany) with excitation at 540 nm and emission at 610 nm. H_2O_2 was used to prepare the standard curve. The reaction mixture without the molecular probe and also without the leaf/root served as control. ROS measurements from leaves and roots were performed using the molecular probe, 5-(and-6)-carboxy-2′,7′-difluorodihydrofluoresceindiacetate (carboxy-H_2DFFDA) according to the manufacturer's instructions (https://tools.invitrogen.com/-content/sfs/ manuals /mp36103.pdf). For ROS measurement, leaf of 0.5-1 mm width and root of 2-3 cm length were incubated in 20 µM carboxy-H_2DFFDA prepared in KRPG buffer consisting of 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$ and 5.5 mM glucose, pH 7.35 for 30 min in dark. The fluorescence intensity was quantified with a fluorescence microplate reader with excitation at 485 nm and emission at 530 nm. The reaction mixture without the molecular probe and also without the leaf/root served as control.

Reverse Transcription-PCR Analysis

Total RNA from the treated Arabidopsis seedlings/roots/shoots were extracted using RNeasy Plant Mini kit with DNAse I treatment (Qiagen). cDNA was synthesised using the Omniscript

cDNA synthesis kit (QIAGEN) using 1 µg RNA. Semi-quantitative RT-PCR was performed using 1:4 fold diluted cDNA. The oligonucleotide primers were designed against the different genes tested (Supplemental Material 5). PCR reactions were allowed to proceed for different number of cycles to determine the exponential phase of amplification. Densitometric analysis of ethidium bromide-stained agarose gels was performed using Quantity One software (Bio-Rad). The mRNA levels for each cDNA probe were normalized with respect to the house keeping gene, *GAPDHC* mRNA levels and also relative to WT control.

ACKNOWLEDGEMENTS

J.M.J. was supported by the IMPRS and FSU Jena. We thank Prof. Scheel for providing flg22; and Sarah Mußbach and Claudia Röppischer for excellent technical assistance.

REFERENCES

Alscher, R.G., Erturk, N. and Heath, L.S. (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Biol*, **53**, 1331-1341.

Anderson, J.C., Bartels, S., Besteiro, M.A.G., Shahollari, B., Ulm, R. and Peck, S.C. (2011) Arabidopsis MAP Kinase Phosphatase 1 (AtMKP1) negatively regulates MPK6-mediated PAMP responses and resistance against bacteria. *Plant J*, **67**, 258-268.

Anderson-Prouty, A.J. and Albersheim, P. (1975) Host-pathogen interactions. VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogens host. *Plant Physiol*, **56**, 286-291.

Asada, K. (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol*, **141**, 391-396.

Blume, B., Nürnberger, T., Nass, N. and Scheel, D. (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defence in parsley. *Plant Cell*, **12**, 1425-1440.

Camehl, I., Drzewiecki, C., Vadassery, J., Shahollari, B., Sherameti, I., Forzani, C., Munnik, T., Hirt, H. and Oelmüller, R. (2011) The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in Arabidopsis. *PLoS Pathog*, **7**, e1002051.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J. and Oelmüller, R. (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. *New Phytol*, **185**, 1062-1073.

Chabaud, M., Genre, A., Sieberer, B.J., Faccio, A., Fournier, J., Novero, M., Barker, D.G., Bonfante, P. (2011) Arbuscular mycorrhizal hyphopodia and germinated spore exudates trigger Ca^{2+} spiking in the legume and nonlegume root epidermis. *New Phytol*, **189**, 347-355.

Deshmukh, S., Hückelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F. and Kogel, K.H. (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proc Natl Acad Sci USA*, **103**, 18450-18457.

Dodd, A.N., Kudla, J. and Sanders, D. (2010) The language of calcium signaling. *Annu Rev Plant Biol*, **61**, 593-620.

Garcia-Brügger, A., Lamotte, O., Vandelle, E., Bourque, S., Lecourieux, D., Poinssot, B., Wendehenne, D., Pugin, A. (2006) Early signaling events induced by elicitors of plant defenses. *Mol Plant Microbe Interact*, **19**, 711-724.

Gechev, T.S., Gadjev, I.Z. and Hille, J. (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants. *Cell Mol Life Sci*, **61**, 1185-1197.

Govrin, E.M., Rachmilevitch, S., Tiwari, B.S., Solomon, M. and Levine, A. (2006) An elicitor from *Botrytis cinerea* induces the hypersensitive response in *Arabidopsis thaliana* and other plants and promotes the gray mold disease. *Phytopathol*, **96**, 299-307.

Heller, J. and Tudzynski, P. (2011) Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. *Annu Rev Phytopathol*, **49**, 369-390.

Hetherington, A.M. and Brownlee, C. (2004) The generation of Ca^{2+} signals in plants. *Annu Rev Plant Biol*, **55**, 401-427.

Hu, X., Neill, S.J., Yang, Y. and Cai, W. (2009) Fungal elicitor Pep-25 increases cytosolic calcium ions, H_2O_2 production and activates the octadecanoid pathway in *Arabidopsis thaliana*. *Planta*, **229**, 1201-1208.

Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., Kogel, K.H. and Schäfer, P. (2011) Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. *Plant Physiol*, **156**, 726-740.

Jeworutzki, E., Roelfsema, M.R.G., Anschütz, U., Krol, E., Elzenga J.T.M., Felix, G., Boller, T., Hedrich, R. and Becker, D. (2010) Early signaling through the Arabidopsis pattern recognition receptors FLS2 and EFR involves Ca^{2+}-associated opening of plasma membrane anion channels. *Plant J*, **62**, 367-378.

Johnson, J.M., Nongbri, P.L., Sherameti, I. and Oelmüller, R. (2011a) Calcium signaling and cytosolic calcium measurements in plants. *Endocyt Cell Res*, **21**, 64-76.

Johnson, J.M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Varma, A. and Oelmüller, R. (2011b) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation - A model system to study plant beneficial traits. *Endocyt Cell Res*, **21**, 101-113.

Kawano, T. and Muto, S. (2000) Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco cell suspension culture. *J Exp Bot*, **51**, 685-693.

Kerchev, P.I., Karpińska, B., Morris, J.A., Hussain, A., Verrall, S.R., Hedley, P.E., Fenton, B., Foyer, C.H. and Hancock, R.D. (2013) Vitamin C and the abscisic acid-insensitive 4

transcription factor are important determinants of aphid resistance in Arabidopsis. *Antioxid. Redox Signal*, **18**, 2091-2105.

Khandelwal, A., Elvitigala, T., Ghosh, B. and Quatrano, R.S. (2008) Arabidopsis transcriptome reveals control circuits regulating redox homeostasis and the role of an AP2 transcription factor1. *Plant Physiol*, **148**, 2050-2058.

Klüsener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V. and Schroeder, J.I. (2002) Convergence of calcium signalling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. *Plant Physiol*, **130**, 2152-2163.

Knight, H., Trewavas, A.J. and Knight, M.R. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J*, **12**, 1067-1078.

Knight, M.R., Campbell, A.K., Smith, S.M. and Trewavas, A.J. **(**1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature*, **352**, 524-526.

Kudla, J., Batistic, O. and Hashimoto, K. (2010) Calcium signals: the lead currency of plant information processing. *Plant Cell*, **22**, 541-563.

Laloi, C., Apel, K. and Danon, A. (2004) Reactive oxygen signalling: the latest news. *Curr Opin Plant Biol*, **7**, 323-328.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R. and Pugin, A. (2002) Analysis and effects of cytosolic free calcium elevation in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell*, **14**, 2627-2641.

Lecourieux, D., Ranjeva, R. and Pugin, A. (2006) Calcium in plant defence-signaling pathways. *New Phytol*, **171**, 249-269.

Lee, Y.C., Johnson, J.M., Chien, C.T., Sun, C., Cai, D., Lou, B., Oelmüller, R. and Yeh, K.W. (2011) Growth promotion of Chinese cabbage and Arabidopsis by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin. *Mol Plant Microbe Interact*, **24**, 421-431.

Lenz, H.D., Haller, E., Melzer, E., Kober, K., Wurster, K., Stahl, M., Bassham, D.C., Vierstra, R.D., Parker, J.E., Bautor, J., Molina, A., Escudero, V., Shindo, T., van der Hoorn, R.A.L., Gust, A.A. and Nürnberger, T. (2011) Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens. *Plant J*, **66**, 818-830.

Marino, D., Dunand, C., Puppo, A. and Pauly, N. (2012) A burst of plant NADPH oxidases. *Trends Plant Sci*, **17**, 9-15.

Matsuo, M., Johnson, J.M., Godfrey, R., Obokata, J., Böhmer, F.D. and Oelmüller, R. (2013) Redox responsive transcription factor1 amplifies the formation of reactive oxygen species in *Arabidopsis thaliana* shoots and roots. *Plant Cell*, under revision.

Maxwell, K. and Johnson, G.N. (2000) Chlorophyll fluorescence - a practical guide. *J Exp Bot*, **51**, 659-668.

Mehterov, N., Balazadeh, S., Hille, J., Toneva, V., Müller-Röber, B. and Gechev, T. (2012) Oxidative stress provokes distinct transcriptional responses in the stress-tolerant *atr7* and stress-sensitive *loh2 Arabidopsis thaliana* mutants as revealed by multi-parallel

quantitative real-time PCR analysis of ROS marker and antioxidant genes. *Plant Physiol Biochem*, **59**, 20-29.

Mithöfer, A. and Mazars, C. (2002) Aequorin-based measurements of intracellular Ca^{2+}-signatures in plant cells. *Biol Proced Online*, **4**, 105-118.

Moebius, N. and Hertweck, C. (2009) Fungal phytotoxins as mediators of virulence. *Curr Opin Plant Biol*, **12**, 390-398.

Mori, I.C. and Schroeder, J.I. (2004) Reactive oxygen species activation of plant Ca^{2+} channels: a signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. *Plant Physiol*, **135**, 702-708.

Navazio, L., Moscatiello, R., Genre, A., Novero, M., Baldan, B., Bonfante, P. and Mariani, P. (2007) A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. *Plant Physiol*, **144**, 673-681.

Nongbri, P.L., Johnson, J.M., Sherameti I., Glawischnig E., Halkier B.A., and Oelmüller, R. (2012). Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between *Arabidopsis* roots and the endophyte *Piriformospora indica*. *Mol Plant Microbe Interact*, **25**, 1186-1197.

Oelmüller, R., Sherameti, I., Tripathi, S. and Varma, A. (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis*, **49**, 1-17.

Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., Hishinuma, H., Senzaki, E., Yamagoe, S., Nagata, K., Nara, M., Suzuki, K., Tanokura, M. and Kuchitsu, K. (2008) Synergistic activation of the Arabidopsis NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. *J Biol Chem*, **283**, 8885-8892.

Oldroyd, G.E., Murray, J.D., Poole, P.S. and Downie, J.A**.** (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet*, **45**, 119-144.

Pedersen, B.P., Kumar, H., Waight, A.B., Risenmay, A.J., Zygy Roe-Zurz, Z., Chau, B.H., Schlessinger, A., Bonomi, M., Harries, W., Andrej Sali, A., Johri, A.K. and Stroud, R.M. (2013) Crystal structure of a eukaryotic phosphate transporter. *Nature*, **496**, 533-536.

Pedras, M.S.C. and Khallaf, I. (2012) Molecular interactions of the phytotoxins destruxin B and sirodesmin PL with crucifers and cereals: Metabolism and elicitation of plant defenses. *Phytochem*, **77**, 129-139.

Peleg-Grossman, S., Melamed-Book, N. and Levine, A. (2012) ROS production during symbiotic infection suppresses pathogenesis-related gene expression. *Plant Sig Behav*, **7**, 409-415.

Peškan-Berghöfer, T., Shahollari, B., Giang, P.H., Hehl, S., Markert, C., Blanke, V., Varma, A.K. and Oelmüller, R. (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. *Physiol Plant*, **122**, 465-477.

Ranf, S., Grimmera, J., Pöschl, Y., Pecher, P., Chinchilla, D., Scheel, D. and Lee, J. (2012) Defense-related calcium signaling mutants uncovered *via* a quantitative high-throughput

screen in *Arabidopsis thaliana*. *Mol Plant*, **5**: 115-130.

Reddy, A.S., Ali, G.S., Celesnik, H. and Day, I.S. (2011) Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *Plant Cell*, **23**, 2010-2032.

Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. and Knight, M.R. (2004) OXI1 kinase is necessary for oxidative burst-mediated signaling in Arabidopsis. *Nature*, **427**, 858-861.

Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell*, **14**, S401-S417.

Schäfer, P., Khatabi, B., Kogel, K.H. (2007) Root cell death and systemic effects of *Piriformospora indica*:a study on mutualism. *FEMS Microbiol Lett*, **275**, 1-7.

Shahollari, B., Vadassery, J., Varma, A. and Oelmüller, R. (2007) A leucine rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. *Plant J*, **50**, 1-13.

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R. (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of Nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. *J Biol Chem*, **280**, 2641-2647.

Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V.M., Nitz, I., Varma, A., Grundler, F.M. and Oelmüller, R. (2008) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. *Plant J*, **54**, 428-439.

Stein, E., Molitor, A., Kogel, K.H. and Waller, F. (2008) Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol*, **49**, 1747-1751.

Sun, C., Johnson, J.M., Cai, D., Sherameti, I., Oelmüller, R. and Lou, B. (2010) *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *J Plant Physiol*, **167**, 1009-1017.

Su'udi, M., Kim, M.G., Park, S.R., Hwang, D.J., Bae, S.C. and Ahn, I.P. (2011) Arabidopsis cell death in compatible and incompatible interactions with *Alternaria brassicicola*. *Mol Cells*, **31**, 593-601.

Takahashi, F., Mizoguchi, T., Yoshida, R., Ichimura, K. and Shinozaki, K. (2011) Calmodulin-dependent activation of MAP kinase for ROS homeostasis in Arabidopsis. *Mol Cells*, **41**, 649-660.

Torres, M.A. (2010) ROS in biotic interactions. *Physiol Plant*, **138**, 414-429.

Toufighi, K., Brady, S., Austin, R., Ly, E. and Provart, N. (2005) The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. *Plant J*, **43**, 153-163.

Vadassery, J., Ranf, S., Drewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J. and Oelmüller R. (2009) A cell wall extract from the endophytic fungus *Piriformospora indica*

promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *Plant J*, **59**, 193-206.

Vadassery, J., Ritter, C., Venus, Y., Camehl, I., Varma, A., Shahollari, B., Novák, O., Strnad, M., Ludwig-Müller, J. and Oelmüller, R. (2008) The role of auxins and cytokinins in the mutualistic interaction between Arabidopsis and *Piriformospora indica*. *Mol Plant Microbe Interact*, **21**, 1371-1383.

Varma, A., Savita Verma, Sudha, S., Sahay, N., Butehorn, B. and Franken, P. (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Appl Environ Microbiol*, **65**, 2741-2744.

Vatsa, P., Chiltz, A., Bourque, S., Wendehenne, D., Garcia-Brugger, A. and Pugin, A. (2011) Involvement of putative glutamate receptors in plant defence signaling and NO production. *Biochimie*, **93**, 2095-2101.

Vidhyasekaran, P., Ruby Ponmalar, T., Samiyappan, R., Velazhahan, R.,Vimala, R., Ramanathan, A., Paranidharan, V. and Muthukrishnan, S. (1997) Host-specific toxin production by *Rhizoctonia solani*, the rice sheath blight pathogen. *Phytopathol*, **87**, 1258-1263.

Wagner, R., Dietzel, L., Bräutigam, K., Fischer, W. and Pfannschmidt, T. (2008) The long-term response to fluctuating light quality is an important and distinct light acclimation mechanism that supports survival of *Arabidopsis thaliana* under low light conditions. *Planta*, **228**, 573-587.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P. and Kogel, K.H. (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc Natl Acad Sci USA*, **102**, 13386-13391.

Yadav, V., Kumar, M., Deep, D.K., Kumar, H., Sharma, R., Tripathy, T., Tuteja, N., Saxena, A.K. and Johri, A.K. (2010) A Phosphate transporter from a root endophytic fungus *Piriformospora indica* plays a role in the phosphate transfer to the plants. *J Biol Chem*, **285**, 26532-26544.

Zhang, Y., Zhu, H., Zhang, Q., Li, M., Yan, M., Wang. R., Wang, L., Welti, R., Zhang, W. and Wan, X. (2009) Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in Arabidopsis. *Plant Cell*, **21**, 2357-2377.

Zhao, J., Devaiah, S. P., Wang, C., Li, M., Welti, R. and Wang, X. (2013) Arabidopsis phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens. *New Phytol*, **199**, 228-240.

Zuccaro, A., Lahrmann, U., Güldener, U., Langen, G., Pfiffi, S., Biedenkopf, D., Wong, P., Samans, B., Grimm, C., Basiewicz, M., Murat, C., Martin, F. and Kogel, K.H. (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. *PLoS Pathog*, **7**, e1002290.

4.5 Manuscript V (The Plant Cell, under revision)

REDOX RESPONSIVE TRANSCRIPTION FACTOR1 amplifies the formation of reactive oxygen species in *Arabidopsis thaliana* shoots and roots

Mitsuhiro Matsuo*, Joy Michal Johnson*, Rinesh Godfrey, Junichi Obokata, Frank-D. Böhmer and Ralf Oelmüller
(*) contributed equally

Supplementary informations are kept as separate files in the attached CD.

REDOX RESPONSIVE TRANSCRIPTION FACTOR1 amplifies the formation of reactive oxygen species in *Arabidopsis thaliana* shoots and roots

Mitsuhiro Matsuo*[1,3], Joy Michal Johnson*[1], Rinesh Godfrey[2], Junichi Obokata[3], Frank-D. Böhmer[2] and Ralf Oelmüller[1**]

[1]Institute of Plant Physiology, Friedrich-Schiller-University Jena, Dornburger Str. 159, 07743 Jena, Germany

[2]Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Jena University Hospital, Jena, Germany

[3] Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan

(*) even contribution

(**) corresponding author; email: b7oera@hotmail.de

Key words: abiotic stress, biotic stress, reactive oxygen species, H_2O_2, redox responsive transcription factor1, RAP2.6

Abbreviations: ABA, abscisic acid; ET, ethylene; JA, jasmonic acid; LL, low light; ML, medium light; HL, high light; LD, long day; SD, short day; oe, overexpressor; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; RRTF1, redox responsive transcription factor1; SA, salicylic acid; WT, wild-type

Running title: RRTF1 amplifies ROS formation

Abstract

Redox Responsive Transcription Factor1 (*RRTF1*) in *Arabidopsis thaliana* is rapidly and transiently upregulated by stress and redox signals. Inactivation of *RRTF1* restricts and overexpression promotes reactive oxygen species (ROS) production in response to biotic and abiotic stress. oe lines are impaired in root and shoot development, light sensitive and susceptible to *Alternaria brassicae* infection. These symptoms are diminished by the beneficial root endophyte *Piriformospora indica* which reduces ROS accumulation locally in roots and systemically in shoots. Approximately 800 stress- redox-, ROS regulated-, ROS

scavenging-, defense-, cell death- and senescence-related genes are regulated by RRTF1, and those genes which are upregulated in all oe tissues are ROS marker genes. Many differentially expressed genes respond to an RRTF1-mediated change in the cellular ROS homeostasis. *RRTF1* is co-regulated with the phylogenetically related *RAP2.6*, but elevated RAP2.6 levels do not promote ROS formation. RRTF1 mediates systemic ROS production in distal non-stressed leaves. We conclude that the highly conserved RRTF1 rapidly, transiently and systemically amplifies ROS formation in response to ROS and ROS producing abiotic and biotic stress signals. Necrotrophs stimulate *RRTF1* expression, while (hemi-)biotrophs and *P. indica* which do not induce ROS production in the host, do not affect or repress *RRTF1* expression.

INTRODUCTION

Plants and many other organisms constantly produce reactive oxygen species (ROS) in chloroplasts, mitochondria, peroxisomes and other sites of the cell because of their metabolic processes such as photosynthesis and respiration (Apel and Hirt, 2004). ROS production is particularly enhanced under environmental constrains and excess ROS production leads to the activation of cell death programs or senescence (Apel and Hirt, 2004; Foyer and Noctor, 2009; Miller et al., 2010; Tripathy and Oelmüller, 2012). Antioxidant enzyme systems and antioxidants such as ascorbate and glutathione detoxify the ROS (Asada, 2006; Dietz et al., 2006) and stabilize the redox poise in the different cellular compartments (cf. Asada, 2000). The entire cellular ROS gene network comprises more than 180 genes in Arabidopsis (Mittler et al., 2004; Mehterov et al. 2012).

ROS also activate specific signaling pathways which counteract ROS-induced cell damage, activate responses to abiotic and biotic stresses or control developmental processes (Jaspers and Kangasjärvi, 2010; Swanson and Gilroy, 2010; Torres, 2010). Pathogen or herbivore attack (Torres, 2010), treatment of plants with pathogen-associated molecular patterns (PAMPs; Mersmann et al., 2010), wounding (Torres and Dangl, 2005), or abiotic stress such as heat (Miller et al., 2009), ozone (Vahisalu et al., 2010) or salt (Torres and Dangl, 2005) induce an oxidative burst, in which plasma membrane-bound NADPH oxidases (respiratory burst oxidase homologues, RBOHs) and cell wall peroxidases release H_2O_2 into the apoplast (Torres and Dangl, 2005). RBOHs are important for defense responses and hormonal signaling (Marino et al., 2012), and are synergistically activated by phosphorylation, binding

of Ca^{2+} to their EF hand motifs and S-nitrosylation (Ogasawara et al., 2008, Yun et al., 2011). Elevated ROS levels in the apoplast are toxic to microbes and play a profound role in mediating rapid, long-distance, cell-to-cell propagating signals by the formation of a ROS wave (Miller et al., 2009; Mittler et al., 2011). Other well characterized ROS-induced responses are closure of stomata (Wang and Song, 2008) and regulation of cell expanse (Carol and Dolan, 2006).

The existence of many interconvertible ROS species makes it difficult to distinguish between cytotoxic and signaling events which are induced by a particular ROS. ROS with signaling functions are H_2O_2, singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$) and superoxide anion radical ($O_2^{\cdot-}$), and a given ROS and its location affect its role in signaling (Wagner et al., 2004; Gadjev et al., 2006; Laloi et al., 2006; Mehterov et al., 2012). Genetic manipulations of ROS generation / scavenging and drug applications have been used to study the effects of particular ROS species. $^1O_2$ is preferentially synthesized in plastids (op den Camp et al., 2003). Paraquat treatment leads to $O_2^{\cdot-}$ (and subsequently H_2O_2) production by an electron flow from photosystem I to oxygen. Peroxisomal catalases detoxify photorespiratory H_2O_2 and CAT2-deficient plants accumulate H_2O_2 in the peroxisomes (Vanderauwera et al., 2005). It is believed that ROS production in a particular cellular compartment can have impacts on ROS levels and signaling in other locations (Gadjev et al., 2006).

Stress-induced ROS-activating responses have to occur rapidly, and should decay when the stress disappears. Here we report on RRTF1, an APETALA2/ethylene response transcription factor (TF) (AP2/ERF). *RRTF1* expression in the nucleus is controlled by signals from the plastids (Khandelwal et al., 2008), induced by jasmonic acid (JA) in a COI1-dependent manner (Wang et al., 2008) and repressed by WRKY40, which binds to the W-box in the *RRTF1* promoter (Pandey et al., 2010). Khandelwal et al. (2008) have shown that RRTF1 is a component of a core redox signaling network that includes EDS1 and WRKY33. However, why such diverse signals regulate *RRTF1* expression and how this is related to RRTF1 function, is not understood. We demonstrate that *RRTF1* expression is rapidly and transiently stimulated by various ROS and ROS-generating biotic and abiotic signals, and that the protein amplifies ROS formation in response to these stimuli. Inactivation of *RRTF1* restricts and overexpression promotes ROS production in response to biotic and abiotic stress signals. We propose that the rapid, transient and local amplification of ROS production may be crucial for activating the appropriate downstream responses to a stimulus.

RESULTS

RRTF1 expression is rapidly up-regulated under stress

To understand how *RRTF1* expression is regulated we compared own data with those from literature and database surveys. Expression of *RRTF1* was rapidly upregulated in response to light stress on plastids. Shifting Arabidopsis seedlings from continuous low (cLL, 30 µmol m^{-2} s^{-1}) to high light (cHL, 200 µmol m^{-2} s^{-1}) for 3 h resulted in an ~4-fold upregulation of the *RRFT1* mRNA level in the leaves, and pretreatment of the leaves with DCMU prior to exposure to cHL completely prevented the induction (Supplemental Table 1A; cf. Khandelwal et al., 2008). The *RRTF1* mRNA levels dropped rapidly when the HL-exposed seedlings were transferred back to LL (Supplemental Table 1B). Database search uncovered that application of methyl viologen, an electron donor of photosystem I, and singlet oxygen generated in the plastids stimulates *RRTF1* expression (Toufighi et al., 2005). Therefore, different stress signals from the plastids control *RRTF1* expression in the nucleus (cf. Khandelwal et al., 2008). Publically available data showed also that *RRTF1* is induced by abiotic stress like salt, drought, cold, UV-B light, heat and osmotic stress (Toufighi et al., 2005; Gadjev et al. 2006; Supplemental Material 1), as well as the stress-related phytohormones ABA (Matsui et al., 2008) and JA (Wang et al., 2008). Furthermore, infection of the leaves with spores of the necrotrophic fungi *Alternaria brassicae*, *Botrytis cinerea*, the necrotrophic bacteria *Pseudomonas syringae* pv. tomato DC3000 and *P. syringae* pv. *phaseolicola* stimulated *RRTF1* expression (Supplemental Material 1). We obtained similar results after application of the PAMPs flg22, chitin or a toxin preparation from *A. brassicae* to the leaves (Supplemental Table 1). The PAMP-induced responses were not prevented by DCMU and are therefore independent of the photosynthetic electron flow. In contrast to necrotrophs, the hemibiotrophic oomycete *Phytophthora infestans* and the biotrophic powdery mildew fungi did not stimulate or even repressed *RRTF1* expression (Toufighi et al., 2005, Pandey et al., 2010, Supplemental Material 1). Cocultivation of Arabidopsis seedlings with the beneficial root-colonizing endophytic fungus *Piriformospora indica* resulted initially in a slight increase in the *RRTF1* mRNA levels in roots and, to a lesser extent, in shoots. After 4 days of cocultivation, the fungus repressed the *RRTF1* mRNA level in both roots and shoots (Supplemental Material 1, cf. Discussion). Taken together, stress and/or redox signals from plastids, abiotic stress and necrotophic pathogens stimulate *RRTF1* expression, while biotrophic and hemibiotrophic pathogens and the beneficial fungus *P. indica* do not affect or

repress *RRTF1* expression. RRTF1 belongs to the subfamily B-3 of the ERF/AP2 TF family (http://www. Arabidopsis.org/index.jsp). The protein is highly conserved in angiosperms and present in the major crop plants (Supplemental Material 2).

35S-RRTF1 overexpressor (oe) lines were light sensitive

RRTF1 was expressed under the control of the 35S promoter (*35S-RRTF1*) in Arabidopsis and three T3 plants with different *RRTF1* mRNA levels (*oe18, oe20* and *oe32*) were compared to the wild-type (WT) and *rrft1*. When the seedlings were grown under continuous middle light (cML, 80 µmol m^{-2} s^{-1}) for 10 days in Petri dishes, the *RRTF1* mRNA levels in the leaves of the *oe18, oe20 and oe32* seedlings were 7- , 13- and 20-fold higher compared to the level in the WT control. No *RRTF1* transcripts could be detected in the knock-out line (Figure 1A).

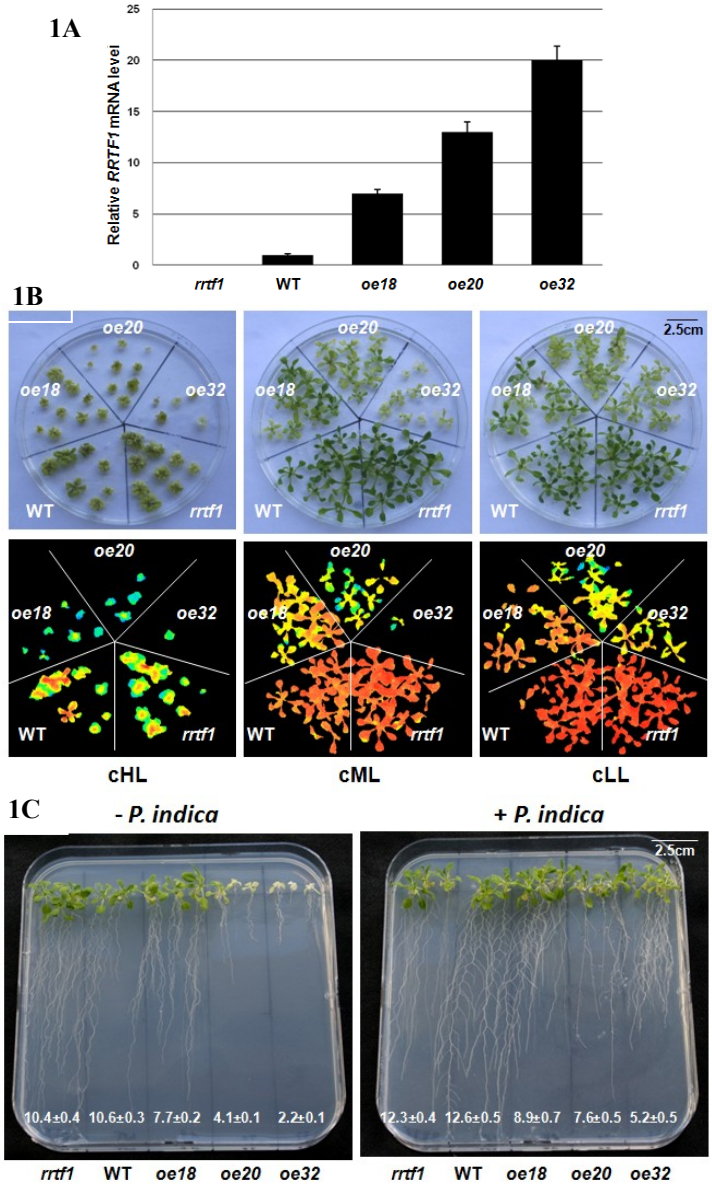
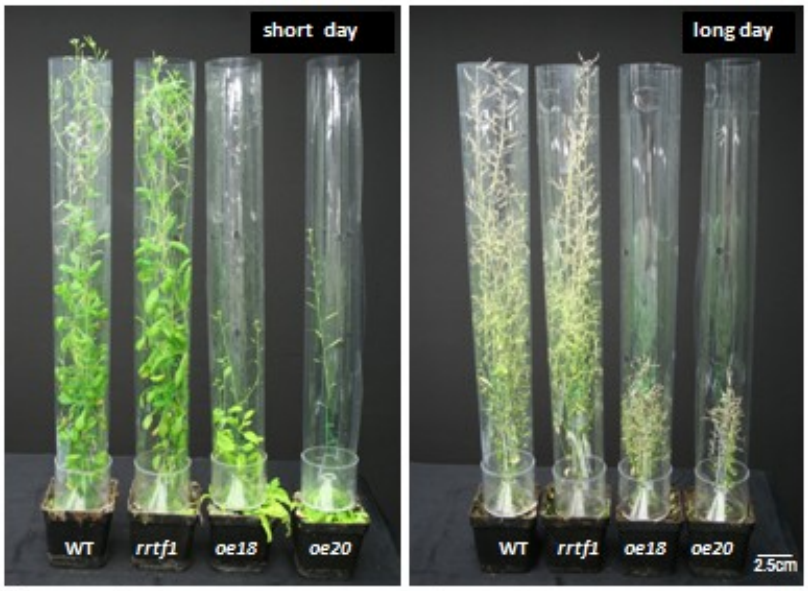


Figure 1. Characterization of *rrtf1* and the *RRTF1* overexpressor lines *oe18*, *oe20* and *oe32*. (A) *RRTF1* RNA levels in the leaves of 10-day old seedlings grown on MS medium in cML. The RNA level of WT seedlings are set as 1.0 and the other levels are expressed relative to it. The qRT-PCR products of *RRTF1* were normalized to those of *GAPDHC* as control. Based on 6 independent qRT-PCR experiments, bars represent SEs. **(B)** 14-day old *rrtf1*, WT, *oe18*, *oe20* and *oe32* seedlings which were grown under cLL, cML or cHL on MS medium. The bottom part shows false color images of the plates representing Fs/Fm values as described in Methods. **(C)** (left): *rrtf1*, WT, *oe18*, *oe20* and *oe32* seedlings grown on Hoagland plates for 18 days in cML and (right) amended with *P. indica* spores as described in Methods. The numbers on the bottom of the plate refer to the lengths of the roots in cm (n=40 seedlings, values are means ± SEs from 4 independent experiments).

The oe lines showed a strong phenotype, when grown in cML for 14 days, and symptoms of photoinhibition and ultimately photobleaching which increased with increasing *RRTF1* expression levels (Figure 1B). Photobleaching was lower under cLL and higher under cHL. After 18 days on Hoagland medium in cML in square plates, the characteristics of the RRTF1-dependent phenotype is even more obvious and *oe32* seedlings were completely white. The leaves of the oe were wrinkled and narrow and the roots were shorter, again in an RRTF1-dependent manner (Figure 1C, left). Therefore, the photosensitivity of the seedlings increased with increase in *RRTF1* expression and light intensity.

The *oe18* and *oe20* seedlings were characterized in more detail. While *oe* seedlings performed relatively well under cLL, the chlorophyll content was reduced compared to WT and *rrtf1* seedlings (Supplemental Material 3). Under cLL, cML and cHL, the photosynthetic parameters ´quantum yield of photosystem II´ (PSII) (Φ_{PSII}), ´photochemical´ (qP), ´non-photochemical quenching´ (NPQ), and ´maximum quantum yield of PSII´ (Fv/Fm) (Supplemental Material 3) of *oe* were decreased with increasing light intensity and *RRTF1* levels. Thus, the efficiency of the photosynthetic electron transport (Φ_{PSII}, qP), the ability of heat dissipation of photochemical energy (NPQ), and the ratio of functional PSII to total PSII (Fv/Fm) was impaired by photoinhibition in the chloroplasts of *oe*. *oe20* seedlings suffered more than *oe18* seedlings. The *rrtf1* seedlings did not show a visible phenotype under these growth conditions when compared to the WT seedlings (Figures 1B and C). After transfer from cLL to cML for 2 days, the stems and emerging young leaves of *oe18* and *oe20* seedlings accumulated large amounts of anthocyanin, while *rrtf1* seedlings grown under the same conditions accumulated less anthocyanin compared to WT (Supplemental Material 3). Taken together, overexpression of *RRTF1* impaired photosynthesis and other plastid functions and induced stress responses in a light-dependent manner.

Fourteen-day old cLL-grown WT, *rrtf1*, *oe18*, *oe20* and *oe32* seedlings were transferred to soil. Almost all oe plants died after 3-8 weeks under cML, but the *oe18* and *oe20* plants survived under long day (LD; 16 h L: 8 h D) ML and even better under short day (SD; 8 h L: 16 h D) ML conditions (Figure 2). Moreover, the biomass and seed weights were significantly decreased with increasing levels of *RRTF1* expression. Interestingly, the biomass and seed weights for *rrtf1* and WT did not differ (Figure 2). Thus, the oe were sensitive to light stress and the survival rate increased with increasing dark incubation periods and lower *RRTF1* levels.



	short day				long day			
	WT	*rrtf1*	*oe18*	*oe20*	WT	*rrtf1*	*oe18*	*oe20*
Survival in soil (%)	100 ± 0	100 ± 0	64 ± 3	43 ± 3	100 ± 0	100 ± 0	46 ± 2	32 ± 2
Seed yield (mg plant^{-1})	123 ± 8	132 ± 6	33 ± 4	20 ± 3	114 ± 4	128 ± 4	47 ± 3	32 ± 3
Dry weight (mg plant^{-1})	480 ± 14	485 ± 14	243 ± 10	186 ± 9	415 ± 15	448 ± 15	179 ± 7	113 ± 5

Figure 2. The *RRTF1* over-expressors survive under light-dark cycles. Fourteen-day old WT, *rrtf1*, *oe18* and *oe2*0 seedlings, grown on MS medium in Petri dishes under cLL, were transferred to soil and grown under SD and LD conditions for 6 weeks. Almost all *oe18* and *oe20* and all *oe32* plants died in cML after 6 weeks on soil and are not shown. The picture shows representative plants under SD or LD conditions. The table gives quantitative data (means ± SEs) based on four independent experiments with 14 plants per line in each experiment. The dry weights were determined from the completely dried plants after harvesting of seeds.

35S-RRTF1 oe lines were highly susceptible to *A. brassicae* infections

Since the *RRTF1* mRNA level was strongly upregulated after *A. brassicae* infection (Supplemental Material 1) and treatment of the leaves with the *A. brassicae* toxin preparation (Supplemental Table 1), we tested whether overexpression of *RRTF1* protected the plants against the pathogen. However, when WT, *rrtf1*, *oe18* and *oe20* leaves were infected with *A. brassicae* spores, the disease symptom development was much faster in oe than WT or *rrtf1* leaves (Figure 3). Quantified data based on "Percentage Disease Index" confirmed that the

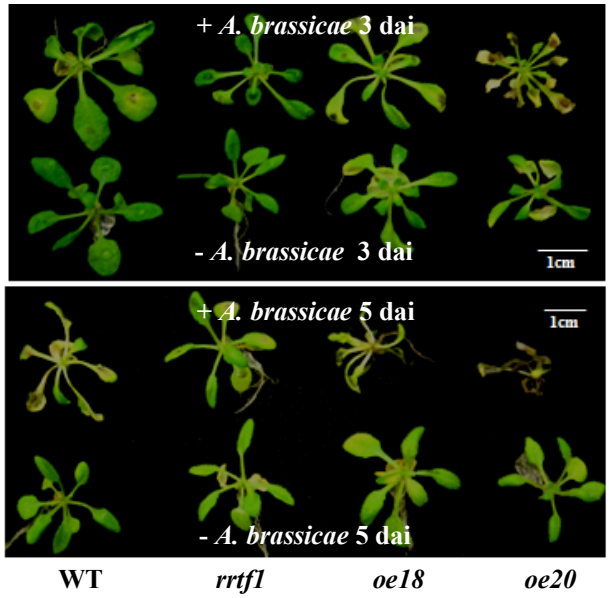
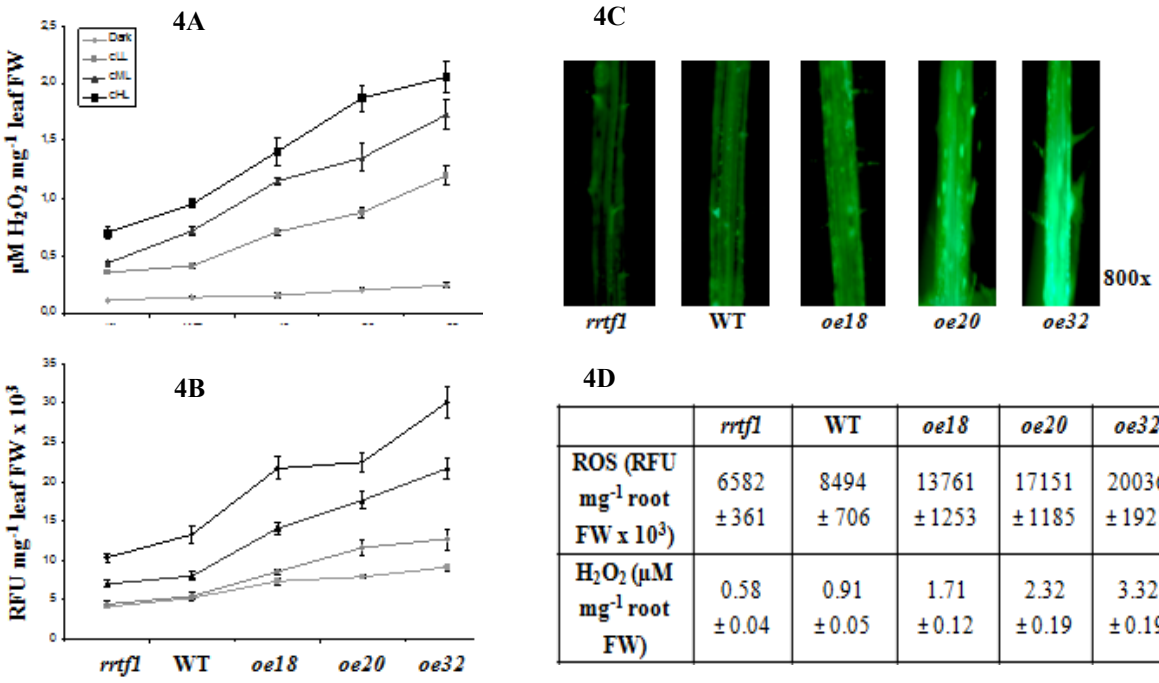


Figure 3. The *RRTF1* overexpressors are highly susceptible to *A. brassicae* infections. 14-day old WT, *rrtf1*, *oe18* and *oe2*0 seedlings, grown in Petri dishes on MS medium under cLL, were transferred to sterile water-soaked Whatmann paper in Petri dishes, and the leaves were inoculated with 5 µl of spore suspension (10^5-10^6 spores ml^{-1}) as described in Methods. Representative pictures were taken at 3 or 5 days after inoculation (dai) from 6 independent experiments with 20 seedlings per line in each experiment. Water was used as control and did not show any effect.

disease development increased with increase of the *RRTF1* expression level (Supplemental Material 4). Therefore, *RRTF1* oe were more sensitive to abiotic (HL) and biotic (*A. brassicae* infection) stress than the WT and *rrtf1*.

RRTF1 promoted ROS formation in leaves and roots

We noticed that staining of the oe with 3,3´-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) was much stronger than WT plants (not shown). This suggested that RRTF1 stimulates H_2O_2 and ROS formation. Quantitative measurements with the Amplex Red reagent (Molecular Probes) confirmed that the H_2O_2 levels in leaves increased with increasing *RRTF1* mRNA levels and light intensity (Figure 4A). Similar results were obtained with the substrate 5-(and-6)-carboxy-2´,7´-difluorodihydro-fluoresceindiacetate (carboxy-H_2DFFDA) (Molecular Probes) which determines the total ROS level (Figure 4B). In all studies, the *rrtf1* knock-out line accumulated less H_2O_2/ROS than the WT (Figures 4A and B). The H_2O_2/ROS levels in dark-adapted leaves of oe seedlings were only marginally higher when compared to the levels in WT and *rrtf1* (Figures 4A and B). This suggests that elevated



4D

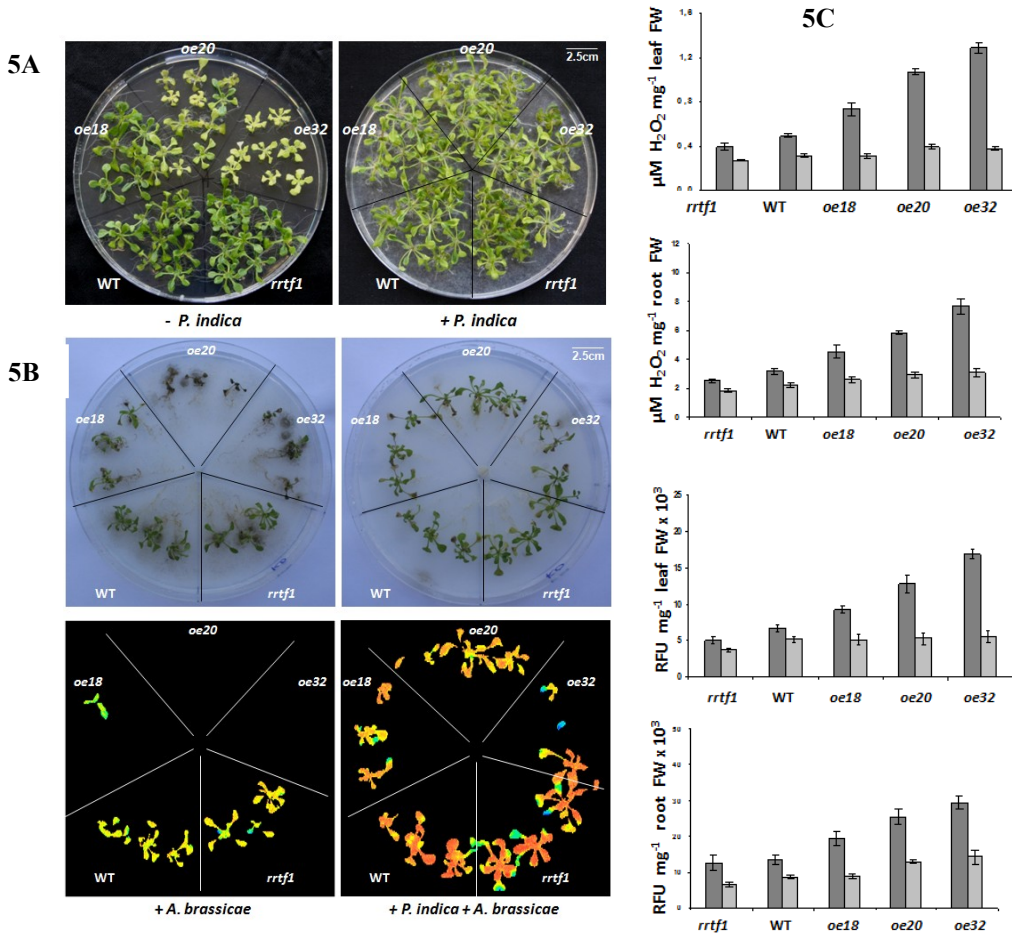
	rrtf1	WT	*oe18*	*oe20*	*oe32*
ROS (RFU mg^{-1} root FW x 10^3)	6582 ± 361	8494 ± 706	13761 ± 1253	17151 ± 1185	20036 ± 1921
H_2O_2 (µM mg^{-1} root FW)	0.58 ± 0.04	0.91 ± 0.05	1.71 ± 0.12	2.32 ± 0.19	3.32 ± 0.19

Figure 4. ROS levels in the leaves (A and B) and roots (C and D) of 14-day old *rrtf1*, WT, *oe18*, *oe20* and *oe32* seedlings. The seedlings were grown in Petri dishes on MS medium in either darkness, cLL, cML or cHL. The total H_2O_2 **(A)** or ROS **(B)** levels were determined for the leaves as described in the Methods. **(C)** The roots were stained with carboxy-H_2DFFDA which determines the total ROS level for imaging. **(D)** Quantified data for H_2O_2 and total ROS levels in the roots is presented in the Table below. All quantified data are based on 6 independent experiments with 20 seedlings per treatment in each line. Mean values ± SEs are given.

ROS production in the oe leaves requires stress signals, and is not caused by higher *RRTF1* expression alone. In contrast, the ROS levels in oe roots were higher irrespective of the light conditions (Figures 4C and D; cf. Discussion). Therefore, several independent assay systems showed that RRTF1 is an important mediator of stress-induced H_2O_2/ROS formation.

P. indica protected the oe against stress by reducing the ROS level

Since beneficial microbes including the root-colonizing endophyte *P. indica* (Baltruschat et al., 2008; Vadassery et al., 2009) stimulate antioxidant systems to counteract ROS-induced damage, we tested whether the stress-exposed oe perform better when the roots were colonized by *P. indica*. As shown in Figures 1C and 5A for seedlings grown under different conditions (Hoagland medium cML and MS medium LD ML), the roots of the oe seedlings cocultivated with *P. indica* are longer, the leaves are bigger and contain more chlorophyll and they are better protected against photoinhibition. *P. indica* also protected the oe leaves against *A. brassicae* infections and the disease development was visibly reduced and/or retarded (Figure 5B). Therefore, the endophyte protects the leaves of the oe lines systemically against abiotic (high light) and biotic (*A. brassicae* infection) stress.



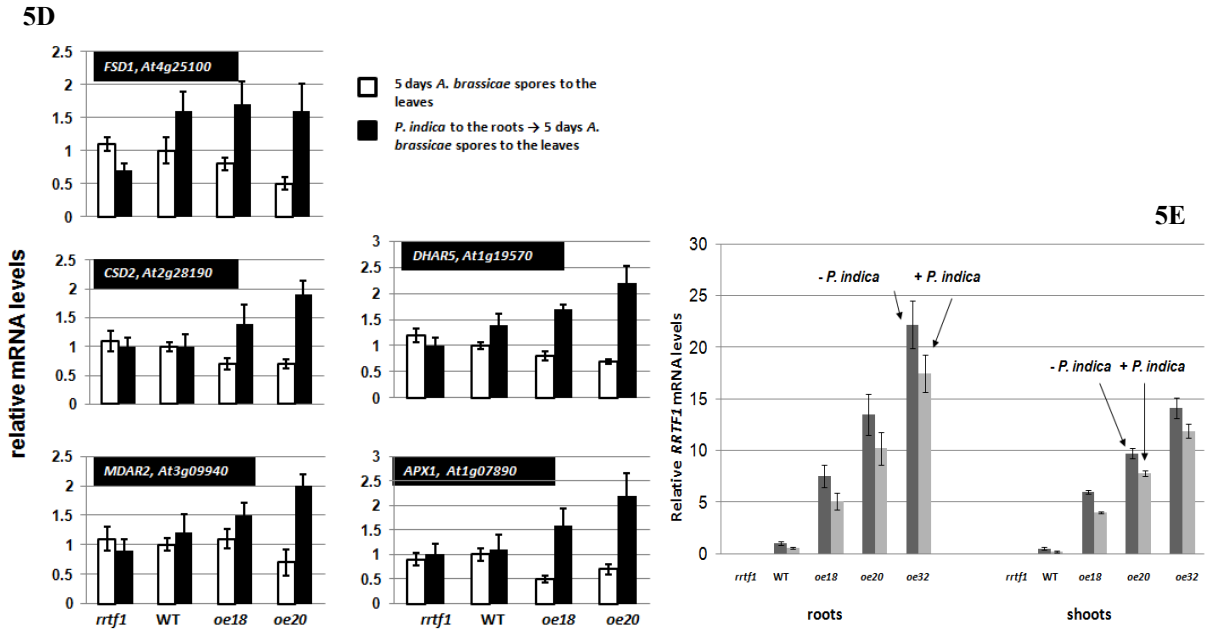


Figure 5. *Piriformospora indica* rescues the light- and *A. brassicae*-induced stress phenotypes of the oe lines by reducing H_2O_2/ROS accumulation in leaves and roots. (A) 14-day old *rrtf1*, WT, *oe18*, *oe20* and *oe32* seedlings grown under LD ML on MS medium, either in the absence (- *P. indica*) or presence (+ *P. indica*) of *P. indica*. Representative pictures from 5 independent experiments with 10 replications in each treatment are shown. **(B)** 12-day old *rrtf1*, WT, *oe18*, *oe20* and *oe32* seedlings grown on MS medium under LD ML were transferred to sterile nylon membrane on PNM plates and each leaf was infected with 5 µl of an *A. brassicae* spore suspension (cf. Methods and Materials). Lower panels: False color images of the plates shown above representing Fs/Fm values as described in Methods. Representative pictures from 5 independent experiments with 10 replications in each treatment are shown. **(C)** H_2O_2/ROS levels in the leaves and roots of 14-day old *rrtf1*, WT, *oe18*, *oe20* and *oe32* seedlings grown under LD ML on MS medium in the absence (dark bars) or presence (light bars) of *P. indica*. Based on 5 independent experiments with 30 seedlings per treatment. Mean values ± SEs are given. **(D)** Relative mRNA levels for ROS scavenging enzymes in the leaves of seedlings which were treated as described under (B). For each panel, the mRNA level of WT seedlings infected with *A. brassicae* was taken as 1.0 (± SEs), and the other values are expressed relative to it. Based on 5 independent experiments with 30 seedlings in each line. **(E)** Relative *RRTF1* mRNA levels in the leaves and roots of 14-day old *rrtf1*, WT, *oe18*, *oe20* and *oe32* seedlings grown under LD ML on MS medium in the absence (dark bars) or presence (light bars) of *P. indica*. Based on 4 independent experiments with 30 seedlings in each line. Mean values ± SEs are given.

The H_2O_2/ROS levels in roots and shoots of *P. indica*-colonized *rrtf1*, WT and oe seedlings were reduced and the degree of the reduction increased with the increase in the H_2O_2/ROS levels present in the roots and shoots (Figure 5C). This was associated with the upregulation of the mRNA levels for ROS scavenging enzymes (Figure 5D) and the downregulation of *RRTF1* (Figure 5E, cf. Discussion). Since the mRNA levels of ROS scavenging enzymes, such as the Fe-superoxide dismutase1 (FSD1), Cu-superoxide dismutase2 (CSD2), monodehydroascorbate reductase2 (MDAR2), dihydroascorbate reductase5 (DHAR5) and ascorbate peroxidase1 (APX1) were not only upregulated in roots (not shown) but also in

shoots of *P. indica*-treated seedlings, the endophyte protected the aerial parts systemically against photoinhibition (Figures 1C and 5A) and disease development (Figure 5B). Better performance of *P. indica*-treated oe seedlings further supports the conclusion that elevated H_2O_2/ROS levels are responsible for the higher stress sensitivity of the oe lines.

RRTF1 regulates stress-, senescence- cell death-, defense- and ROS-related genes in adult plants

Under SD ML, the young *oe18* and *oe20* plants performed quite well. Although their leaves were slightly smaller than WT leaves after transfer to soil for 4 weeks (Supplemental Material 5), they did not show visible symptoms of photodamage and the photosynthetic parameters did not differ significantly from those of WT (data not shown). The ROS levels in *oe18* leaves were 1.6 ± 0.4-times and those in *oe20* leaves were 2.1 ± 0,6-times (n=6) higher than in WT leaves. A comparative transcriptome analysis of *oe18* and WT leaves from three independent experiments was performed. Compared to WT, 588 genes were upregulated and 231 genes downregulated at least 2-fold in *oe18* leaves (Supplemental Material 6A). Categorization of the RRTF1-regulated genes using the Mapman software revealed that most of them code for TFs (19 for APETALA2/ET-responsive element binding proteins (AP2/EREBP); 19 for MYB and 12 for WRKY TFs, 10 for basic helix-loop-helix family members; Supplemental Material 6B). Genes for defense- and stress-related proteins, senescence and cell death proteins and redox regulators were also regulated in *oe18* leaves. In all categories, genes with ROS-related functions were present (cf. Discussion). For a few genes the microarray results were confirmed by real-time and qRT-PCR analyses (Figure 6). The results confirmed the microarray data and supported the concept that RRTF1-mediated elevation of the ROS level in the *oe18* leaves was responsible for the regulation of these genes (cf. Discussion).

Based on studies by Vanderauwera et al. (2005), Gadjev et al. (2006) and Mehterov et al. (2012), the ROS regulated genes can be classified as those preferentially regulated by H_2O_2, $O_2^{\cdot-}$, $^1O_2$ or as common ROS marker genes. To test whether specific ROS marker genes were regulated in *oe18*, the published microarray data were compared with our data sets (Supplemental Material 7). In *oe18* leaves, 4 of the 53 H_2O_2, 3 of the 18 $O_2^{\cdot-}$, 6 of the 22 $^1O_2$ and 17 of the 87 common ROS marker genes were upregulated. Only one H_2O_2 and two common ROS marker genes were downregulated in *oe18* leaves relative to WT control. Therefore, no obvious preference for any of the ROS species could be detected among the ROS regulated genes in the oe line.

Transcriptome analyses of shoots and roots of *rrtf1*, WT and *oe18* seedlings grown under short day LL

The elevated ROS level in the leaves over a period of 4 weeks might cause severe alterations in developmental processes and thus gene expression. To identify more direct *RRTF1* target genes we compared the expression profiles of the shoots and roots of young *oe18* and *rrtf1* seedlings with WT controls, and with the expression profiles from the mature *oe18* leaves (Supplemental Material 6). To minimize photodamaging effects, the seedlings were grown under SD LL condition for 12 days (Supplemental Material 5). The original data and initial analysis are presented in Supplemental Material 8. The overall analysis supports the conclusion from the adult plants that ROS-regulated genes are upregulated in *oe18* seedlings (cf. Discussion). Also similar to the results obtained for the adult leaves, we could not detect any preference for genes responding to a particular ROS species in the cotyledons or roots.

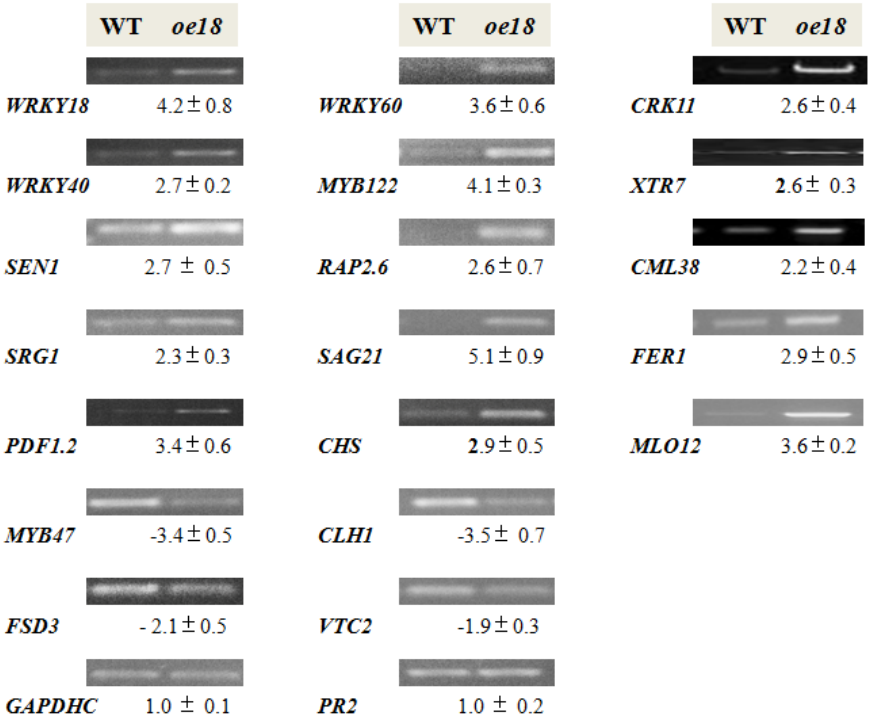


Figure 6. Genes which are differentially regulated in the leaves of WT and *oe18* plants. The plants were grown under SD cML conditions on soil for 4 weeks. The pictures show typical RT-PCR images. Quantified data based on 6 independent qRT-PCR analyses are given below. The values represent fold change of the *oe18* mRNA level relative to the WT control. SEs are the sum of the individual SEs. *GAPDHC* served as housekeeping gene.

However, when the expression profiles of the leaves from adult plants were compared with those from the cotyledons and roots of the seedlings, little overlap could be detected (Figure 7

and Supplemental Table 2). This might be caused by developmental or organ differences, or by the fact that the younger material was not exposed to elevated ROS levels over a longer period. Only 11 genes were upregulated in all three *oe18* tissues, but they are all classified as ROS marker genes (Figure 7 and Supplemental Table 2). Thirty-one genes were upregulated in *oe18* leaves and *oe18* cotyledons and 25 of them were classified as ROS marker genes (Supplemental Table 2 and Supplemental Material 9). Among the genes which are upregulated in *oe18* roots was *OXI1* (Supplemental Material 9) which codes for a well-characterized kinase regulated by H_2O_2 (Rentel et al., 2004). Overall, from 41 genes upregulated in more than one *oe18* tissue, 31 are associated with ROS functions (Figure 7, Supplemental Table 2 and Supplemental Material 9). The list includes genes for H_2O_2 producing enzymes such as myo-inositol oxygenase (At2g19800), peroxidases (At2g37130, At5g64120) and a diphenol oxidase (At5g01040/At5g01050), the dehydrogenase At3g30775 which is localised in the inner membrane of mitochondria (Funck et al., 2010), At1g63040 and At2g21650, two chloroplast regulated nuclear localized TFs (Balazadeh et al., 2012), At4gg13660, At4g34230, At5g13930 and At5g05600 involved in flavanoid and phenylpropanoid biosynthesis (Kimura et al., 2003, Nakatsubo et al., 2008, Bohmer and Schroeder 2011, Fujita et al., 2012) and At3g48520, a negative regulator of the JA signaling cascade (Heitz et al., 2012). None of the genes which are downregulated in more than one *oe18* tissue is classified as ROS marker gene (Figure 7).

The number of *RRTF1*-responsive genes in the cotyledons was higher than that in the roots (Supplemental Material 8). Interestingly, the majority of the genes which were up- (down-) regulated in *oe18* cotyledons or roots were also up- (down-) regulated in *rrtf1* cotyledons or roots relative to the WT control (Supplemental Material 8). Apparently, their expression was not directly regulated by the amount of RRTF1 present in the insertion, WT and overexpression lines (cf. Discussion). Only two genes were > 2-fold upregulated in *oe18* cotyledons and roots and > 2-fold downregulated in *rrtf1* cotyledons and roots: *RAP2.6* (At1g43160) and the gene for a leucoanthocyanin dioxygenase (At3g55970). In summary, the comparative expression profile analyses confirmed that many well-known ROS responsive genes involved in quite different ROS responses are regulated in the leaves, cotyledons and roots of the *rrtf1*. We could not detect any specificity for a particular ROS species. The data suggest that the regulated genes respond to RRTF1-induced cellular changes, likely associated with the ROS homeostasis (cf. Discussion).

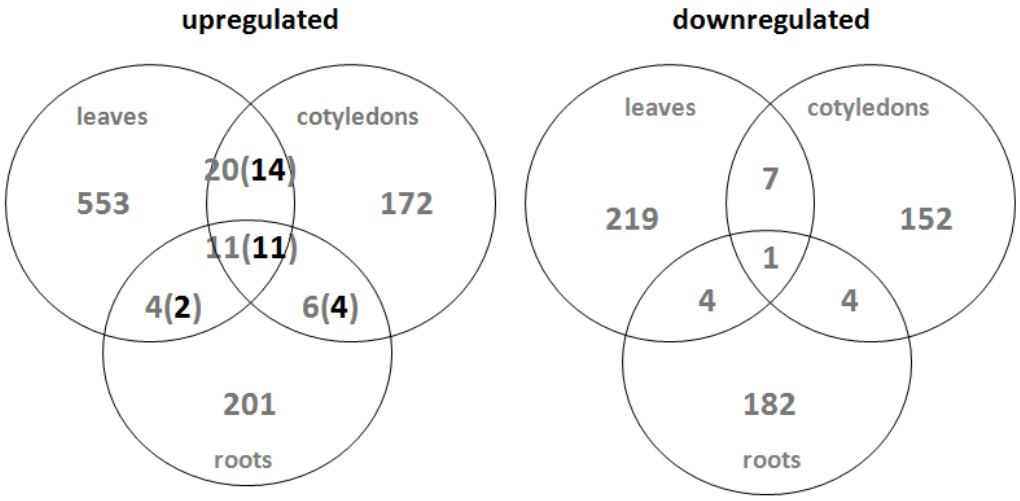


Figure 7. ROS-regulated genes are upregulated in oe. Venn diagram of the number of genes differentially expressed (upregulated (left), downregulated (right)) in *oe18* leaves of adult plants, *oe18* cotyledons and *oe18* roots of seedlings, relative to the wild-type controls. For genes which are upregulated in more than one organ, the numbers in brackets refer to those with ROS related functions. They are presented in Supplemental Table 2. None of the genes which are downregulated in more than one organ shows ROS related functions.

RRTF1 and RAP2.6

The similar regulation of *RRTF1* and *RAP2.6* in our expression profiles (Supplemental Material 8), the close phylogenetic relationship of the two AP2/EREBP TFs (Dietz et al., 2010, Nakano et al. 2006), the observations that both are responsive to the same stress hormones and abiotic stresses, and that their oe lines are smaller under greenhouse conditions and light sensitive (Krishnaswamy et al., 2011), prompted us to test whether both proteins have similar or related functions. When seedlings overexpressing either *RRTF1* or *RAP2.6* were grown under identical conditions, the *RRTF1* oe lines were more light-sensitive than the *RAP2.6* oe lines (Figure 8A). Furthermore, we did not detect elevated H_2O_2 or ROS levels in the *RAP2.6* oe lines (Figure 8B). Finally, the *RAP2.6* mRNA level increased with increase in *RRTF1* overexpression, but the *RRTF1* mRNA level did not increase with increase in *RAP2.6* overexpression (Supplemental Material 10). However, the *RRTF1* mRNA level was strongly upregulated in the *rap2.6* insertion line in roots and in particular in shoots (Supplemental Material 10). This suggests that *RRTF1* expression is either upregulated to compensate for the loss of *RAP2.6* expression, or *rap2.6* plants are stressed and therefore upregulate *RRTF1* expression. These results demonstrate that RRTF1 and not RAP2.6 is responsible for ROS amplification.

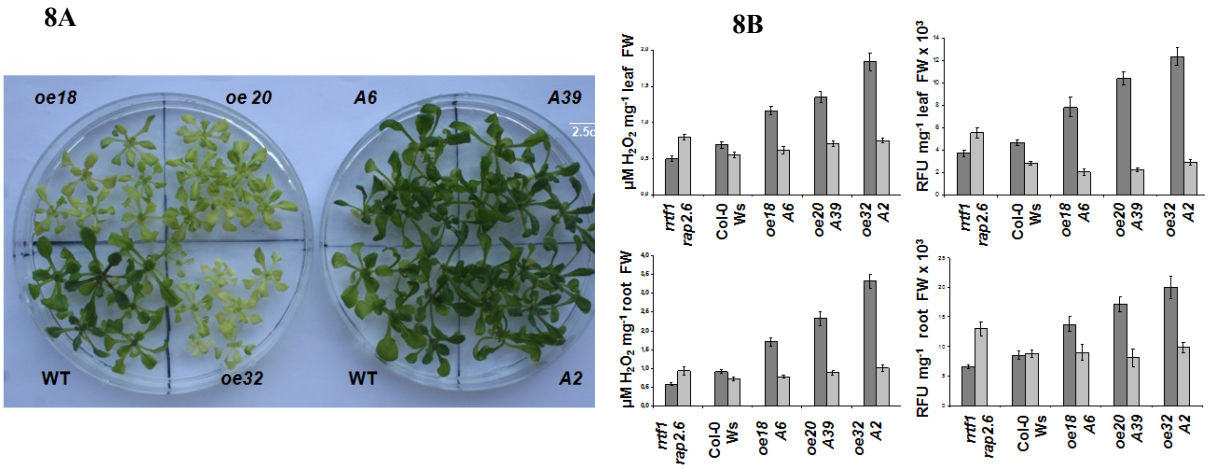


Figure 8. Comparison of *RRTF1* and *RAP2.6* overexpressor lines. (A) WT, *RRTF1* (*oe18*, *oe20*, *oe32*) and *RAP2.6* (*A6*, *A39* and *A2) oe* lines were grown for 10 days under cLL before transfer to cML for 5 days. **(B)** Roots and shoots of these seedlings were harvested separately for H_2O_2/ROS determination as described in Methods. The data are means of 5 independent experiments with 40 seedlings in each line, bars represent SEs. *A6*, *A39* and *A2* are in the Wasilewski (Ws) background and Ws WT was used as control.

RRTF1 is involved in systemic ROS production

Arabidopsis WT and *rrtf1* plants were grown on soil for 4 weeks under SD ML conditions, before half parts of the plant were exposed to cHL, the other parts were covered with aluminum foil (Supplemental Material 11). After 3 and 6 days, we observed a systemic, ~4-fold upregulation of the *RRTF1* mRNA in the distal, shaded leaves of WT plants (Supplemental Table 3). Also the ROS level increased ~2-3-fold in the shade-exposed leaves of WT plants. For *rrtf1* plants, the ROS level in the distal leaves increased ~10 (Supplemental Table 3). When RRTF1-responsive genes (*Myb122*, *CML38*, *PDF1.2*, *WRKY40*, cf. Figure 6) were analysed 3 days after exposure of the plants to cHL, they were significantly upregulated in the shaded leaves of WT, but not *rrtf1* plants (Supplemental Table 3). After 18 days, the number of leaves with severe visible lesions in photosynthesis and chlorophyll accumulation in the shaded areas was 6 times higher in WT than in *rrtf1* plants (Supplemental Table 3). The ROS level in the shaded WT leaves was twice as high as the level in the shaded *rrtf1* leaves (Supplemental Table 3). When senescence-associated ROS-regulated genes (*SEN1*, *SRG1*, *SAG21*) and the stress-related chalcone synthase (*CHS*) gene were analysed, none of them were significantly upregulated in shaded leaves of *rrtf1* leaves, while they were significantly higher in the shaded leaves of WT leaves (Supplemental Table 3). These findings suggest that RRTF1 participates in the systemic regulation of ROS accumulation.

DISCUSSION

RRTF1 amplifies ROS formation

We demonstrate that the highly conserved *RRTF1* (Supplemental Material 2) is rapidly and transiently (Supplemental Table 1B) upregulated in response to abiotic (HL) and biotic (*A. brassicae* infection) H_2O_2/ROS-inducing stresses. Database search also demonstrates that various ROS-inducing abiotic stresses (Jaspers and Kangasjärvi, 2010), ozone which promotes apoplastic H_2O_2 formation (Vahisalu et al., 2010; Wrzaczek et al., 2010), necrotrophic fungi (Supplemental Table 1, Supplemental Material 1) which generate H_2O_2 (Heller and Tudzynski, 2011), PAMPs such as flg22 and chitin which generate $O_2^{\cdot-}$ and subsequently H_2O_2 (Nürnberger et al., 1994; Lamb and Dixon, 1997) and the stress-related and ROS-inducing phytohormones JA and ABA (Matsui et al., 2008; Wang et al., 2008) stimulate *RRTF1* expression. High *RRTF1* mRNA levels are detectable in *flu* mutant in the light which accumulates $^1O_2$ in plastids (op den Camp et al., 2003, Gadjev et al., 2006), in mutants which accumulate ROS due to genetic manipulation of ROS scavenging systems in plastids, mitochondria, peroxisomes or the cytosol (Gadjev et al., 2006; Balazadeh et al., 2012; Mehterov et al., 2012), and in the *JUNGBRUNNEN1* oe, which accumulates H_2O_2 due to the overexpression of a H_2O_2-inducible NAC TF (Wu et al., 2012). Thus, *RRTF1* is induced by different types of ROS from different cellular compartments. ROS are signaling molecules for cell-to-cell communication, and RRTF1 participates in systemic responses to various stress signals (Supplemental Table 3; Rossel et al., 2007; Miller et al., 2009). We propose that RRTF1 amplifies ROS formation and thus ROS signaling in response to ROS-generating stress (Figure 9). This also spreads the ROS signal systemically and activates stress responses in distal not yet stress exposed areas. WRKY40 is a repressor of *RRTF1* (Pandey et al., 2010) and may be involved in the rapid downregulation of *RRTF1* expression if a stress disappears (Supplemental Table 1B). *WRKY40* and other *WRKY* genes are also upregulated in *oe18* plants (Figure 6), which suggests that stressed and ROS-accumulating plants try to restrict *RRTF1* expression and thus ROS production via a feedback loop to prevent excess ROS formation (cf. Figures 6 and 9).

The stress-sensitivity of oe (light: Figures 1B, 1C left, 2 and 5A left, *A. brassicae* infection; Figures 3 and 5B) clearly demonstrates that control of *RRTF1* expression is important for the plants, since the oe accumulate harmful amounts of ROS even when exposed to mild stress conditions (Figures 4A and B). ROS accumulation in the oe lines occurs in the light and not or

to a lesser extent in the dark (Figures 4A and 6B). Thus, elevated *RRTF1* expression *per se* does not lead to higher ROS levels and the TF must be post-transcriptionally activated to generate ROS. The insertion line performs better than the WT, which suggests that ROS amplification is not always required to counteract stress. However, rapid and transient establishment of local ROS maxima under extreme stress such as high light (600 µmol m^{-2} s^{-1}; Khandelwal et al., 2008) may require RRTF1 to activate appropriate stress responses.

RRTF1-regulated genes

To identify target genes of RRTF1, we compared several microarray sets (*oe18 vs*. WT adult leaves; *oe18 vs*. WT *vs. rrtf1* cotyledons; *oe18 vs*. WT *vs. rrtf1* roots). The analysis did not discover any specificity/preference for one of the bioactive ROS species (Supplemental Material 7-2) or for a cellular compartment of the protein (Supplemental Materials 6B-2 and 8-2; cf. Gadjev et al., 2006; Wrzaczek et al., 2010). All datasets identified genes which are directly or indirectly regulated by H_2O_2/ROS. Depending on the organ, tissue, the developmental stage and age of the plant material, the genes are involved in different ROS-regulated processes. ROS inducing-, stress- and defense-related genes are preferentially upregulated in the *oe18* cotyledons and roots (Supplemental Table 2, Supplemental Materials 8-2 and 9).

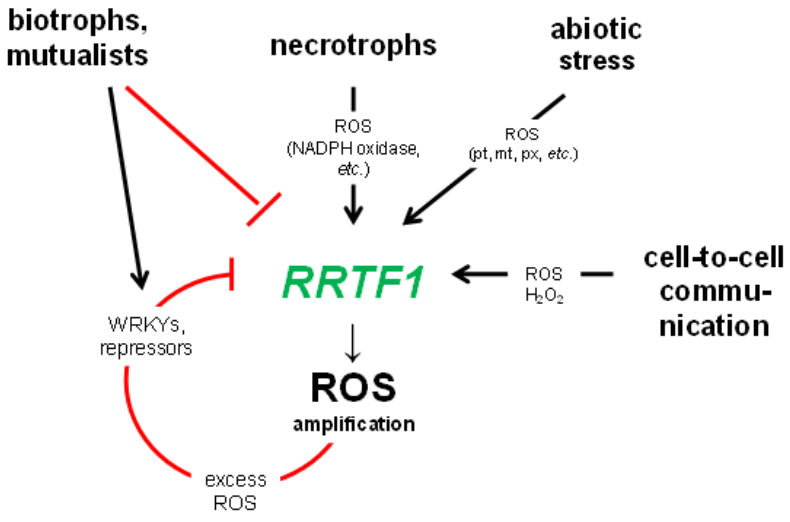


Figure 9. A model describing the role of RRTF1 in ROS amplification. Black arrows show ROS-generating stimuli which trigger *RRTF1* expression; red lines show stimuli which repress *RRTF1* expression.

In the leaves of adult *oe18* plants, senescence and cell death associated genes are also upregulated (Figure 6 and Supplemental Material 6B-2). Genes for C_2H_2, zinc finger, AP2/ERF, Myb and WRKY TFs are the most dominant class of regulated genes. Zat10 (At1g27730), a C_2H_2 zinc finger TF, increases the resistance to photooxidative stress (Rossel

et al., 2007). RAP2.6 (At1g43160), an AP2/EREBP TF which is phylogenetically related to RRTF1 (Nakano et al., 2006; Dietz et al., 2010), participates in ABA, salt, drought and osmotic stress responses and is responsive to JA, SA, ABA and ET (Zhu et al., 2010; Krishnaswamy et al., 2011). RRTF1 and RAP2.6 are part of an AP2/EREBP-linked gene network which coordinates the plant response to redox changes, organelle to nucleus retrograde signals and various stresses (Dietz et al., 2010). Six (DREB-21910, DREB-77640, TEM1, RAP2-4b, RAP2.6 and RAP2.8) of the proposed 21 network members are differentially regulated in *oe18*. ROS, in particular $^1O_2$, play a crucial role in plastid-to-nucleus signaling (Galvez-Valdivieso and Mullineaux, 2010). The integration of the plastid-derived ROS signals with those from various other stress-induced ROS signals through the amplification by RRTF1 suggests that there might be an intensive crosstalk.

Several of the identified *MYB* genes function in stress or defense responses, are related to ROS signaling and protect plastids from stress and photodamage (Saibo et al., 2009). MYB28, MYB29 and MYB76 are regulators of aliphatic glucosinolates (Sønderby et al., 2010) and MYB59 which is repressed by H_2O_2 to promote root hair growth (Mu et al., 2009) is downregulated in *oe18*. The regulated *WRKY* genes are involved in hormone-induced biotic and abiotic responses or related to ROS functions including cell death programs. WRKY40 binds to the W-box in the promoter of *RRTF1* to repress many genes involved in plant defense (Pandey et al., 2010). Stimulation of *WRKY40* in *oe18* (Figure 6) may indicate that RRTF1 controls the expression of its own repressor via ROS. WRKY18, WRKY40 and WRKY60 are induced in responses to ABA, abiotic stress (Chen et al., 2010) and microbial pathogens (Xu et al., 2006). H_2O_2-regulated WRKY53 and WRKY6 control defense and leaf senescence (Miao and Zentgraf, 2010; Robatzek and Somssich, 2001). This confirms that RRTF1 interferes with the regulatory circuits of the WRKY TFs (cf. Khandelwal et al., 2008; Pandey et al., 2010; Figure 6, Supplemental Materials 6B-2 and 8-2). Finally, the JA- and ET-regulated *PDF1.2* but not the SA-regulated *PR-2* was induced in the oe (Figure 6). Therefore, RRTF1 appears to be more involved in JA/ET- than in SA-mediated stress responses.

Genes for major ROS producers such as the plasma membrane-localized NADPH oxidases, apoplastic peroxidases, peroxisomal glycolate oxidases and the components of the photosynthetic electron transport chain were not upregulated, and some genes for the mitochondrial respiration (e.g. β and γ subunits of ATP synthase, cf. Supplemental Material 6B-2) were even downregulated in *oe18* compared to the WT leaves. From ~150 genes for

components of the ROS scavenging network (Mittler et al., 2004), 17 are regulated in *oe18* leaves, and seven of them code for members of the glutaredoxin gene family (cf. below; Supplemental Material 6B-2). They are located in chloroplasts/mitochondria (At1g03850, At5g11930), the cytosol (At4g33040, At362930), and the apoplast (At4g15700, At3g62950, At2g47880). Glutarredoxins in the different cellular compartments together with a cytoplasmic (At1g45145) and a plastid-localized (At5g61440) thioredoxin and the ascorbate-synthesizing VTC2 protein are involved in redox regulation (Rouhier 2010). Apparently, the elevated ROS level in *oe18* induces preferentially gluthatione-sensitive glutaredoxin genes and also other genes involved in redox regulation, e.g. for dehydroascorbate reductase 2, ascorbate oxidase, vitamin c defective 2, the iron superoxide dismutase 3 and two thioredoxins (Supplemental Materials 6B-2 and 8-2).

In addition, several senescence- and programmed cell death associated genes such as *SEN1* or *SRG1* are constitutively upregulated in *oe18* (Figure 6 and Supplemental Material 6B-2). Interestingly, *OXI1* which has been identified as a H_2O_2-inducible serine kinase gene (Rentel et al., 2004) is induced in *oe18* and slightly repressed in *rrtf1* roots. Taken together, many RRTF1-regulated genes are directly or indirectly related to ROS function or homeostasis. Upregulation of genes involved in pathogen defense in shaded distal leaves of cHL-exposed plants suggests that part of the produced ROS is released into the apoplast. How they are perceived is unknown at present. We found that *CRK11* which encodes a cysteine-rich receptor-like kinase in the plasma membrane is upregulated in *oe18*. *CRK11* is also regulated in response to ROS, PAMPs, light stress and ozone, which is ultimately converted to H_2O_2 in the apoplast (Wrzaczek et al. 2010, and references therein). Therefore, CRK11 may be a candidate to sense ROS through redox modification in its extracellular domain.

The vast majority of the genes is up- (down-) regulated in the microarrays irrespective of whether RRTF1 is inactivated (*rrtf1*) or overexpressed (*oe18*) (Supplemental Material 8-2) are not direct targets of RRTF1. This becomes obvious by comparing the expression profiles of *rrtf1* cotyledons (roots) with *oe18* cotyledons (roots) relative to the WT controls. This suggests that the identified genes respond to downstream events which are induced by alterations in RRTF1 levels. Likewise comparison of young cotyledons and adult plants do not show much overlap in the regulated genes. This suggests that long-term accumulation of RRTF1 in the leaves leads to developmental alterations due to constitutively high ROS levels. *RAP2.6* provides an interesting exception: *RAP2.6* is one of two genes which is upregulated in

the roots and shoots of *oe18* and downregulated in roots and shoots of *rrtf1*. Therefore, these genes are either directly regulated by RRTF1 or co-regulated with *RRTF1*. RRTF1 and RAP2.6 are phylogenetically related and their expression is regulated by stress signals and stress hormones (Nakano et al., 2006, Dietz et al., 2010; Krishnaswamy et al., 2011). oe for both genes have a dwarf phenotype (Figure 2; Krishnaswamy et al., 2011) and flower earlier in the greenhouse, and young leaves are often wrinkled. To check the relation between the two TFs, we grew *RAP2.6* oe (Krishnaswamy et al., 2011) under the same conditions as *RRTF1* oe. Figure 8A demonstrates that only the *RRTF1*, but not the *RAP2.6* oe show a bleached phenotype. Furthermore, the *RAP2.6* oe lines did not accumulate higher ROS levels (Figure 8B). This suggests that RAP2.6 alone does not control ROS accumulation.

***RRTF1* regulation by necrotrophic, biotrophic and beneficial microbes**

Database analyses (Supplemental Material 1) and infection studies with *A. brassicae* and *P. indica* (Figure 5E, Supplemental Table 1 and Supplemental Material 1) support the concept that necrotrophs stimulate *RRTF1* expression and thus ROS production, while biotrophs and beneficial microbes, which live and propagate in living host cells, either do not activate or repress *RRTF1* expression. Interestingly, cocultivation of Arabidopsis with *P. indica* results in an initial upregulation of the *RRTF1* mRNA level, while the message level is downregulated during later stages of the symbiosis (Supplemental Material 1). Likewise, during early stages of mycorrizha formation, H_2O_2 is produced and this production declines as soon as a mutualistic interaction has been established (Fester and Hause, 2005). Regulation of *RRTF1* in *P. indica*-colonized plants occurs in roots and leaves (Figure 5E) indicating a systemic effect of the fungus on *RRTF1* expression. The *RRTF1* promoter contains more than 10 W boxes (Pandey et al., 2010) which are putative binding sites of WRKY TFs (Yamasaki et al., 2012), and Pandey et al. (2010) have demonstrated that WRKY40 represses *RRTF1* expression by binding to its promoter. *WRKY18*, *WRKY33*, *WRKY40* and *WRKY60* are induced in response to H_2O_2/ROS and H_2O_2/ROS generating agents (https://www.genevestigator.com, and references therein). WRKY18 and -40 act in a feedback repression system controlling basal defense in Arabidopsis against powdery mildew infection (Pandey et al., 2010). The transcript levels of WRKY18, -33, -40 and -60 are also upregulated in *oe18* (Figure 6, Supplemental Materials 6B-2 and 8-2). This suggests that *RRTF1* expression is controlled by a feedback regulation, in which excess H_2O_2/ROS represses *RRTF1 via* WRKY TFs (Figure 9). Consistent with this idea, the biotrophic

pathogen *G. orontii* stimulates *WRKY40* expression to repress *RRTF1* transcription (Pandey et al., 2010) and thus excess ROS formation. We propose a model, in which ROS-generating stimuli such as necrotrophs, abiotic stress or cell-to-cell communication signals stimulate *RRTF1* expression. This results in the amplification of ROS generation. RRTF1 activates WRKY40 and other WRKY TFs which repress RRTF1 expression and thus prevent excess ROS formation. Biotrophic and mutualistic microbes repress RRTF1 expression either directly or via the activation of a WRKY repressor (Figure 9). Another interesting observation is that *P. indica* actively represses ROS accumulation by activating ROS scavenging genes. This is particularly striking for the oe lines. Figures 1C, 5A and 5B demonstrate that stress-exposed oe lines can better survive in the presence of *P. indica*, and this is associated with a strong reduction of the ROS level and the activation of the ROS scavenging genes (Figures 5C and D). The slight reduction of ROS level in the stress-exposed *rrtf1* in the presence of *P. indica* demonstrates that some reduction of ROS also occurs independently of RRTF1 (Figure 5C). In colonized WT, the lower ROS level is mediated by the activation of ROS scavenging systems and the downregulation of *RRTF1* (Figure 5E, Supplemental Material 1). Therefore, better performance of stress exposed plants by *P. indica* is caused by two strategies: repression of ROS accumulation by activating ROS scavenging systems and repressing *RRTF1* expression (Figure 9).

In summary, we demonstrate that RRTF1 amplifies ROS production in response to biotic and abiotic stress signals. This might be important for a rapid and transient establishment of local ROS maxima to induce appropriate downstream responses. Constitutive upregulation of *RRTF1* expression has severe consequences since even minor stress induces ROS levels which are harmful for the oe. The tight regulation of *RRTF1* suggests that the TF is important in controlling ROS formation. How a TF can control H_2O_2/ROS levels remains unknown, however, the nuclear location (not shown), domain structure (Supplemental Material 2), putative DNA-binding domains and putative interaction partners of RRTF1 suggest that this process is initiated at a transcriptional level.

METHODS

Plant material and growth

Arabidopsis thaliana Col-0 (for Fig. 10B, Ws) was used as WT control. The T-DNA-inserted *RRTF1* knockout mutant (SALK_150614) was obtained from NASC (http://arabidopsis.info/).

Homozygote *RRTF1* knockout plant was identified by PCR with gene specific primers (LP_SALK_150622: 5'-CGCGATGCTTTGTAGGAGTAG-3', RP_SALK_150622: 5'-TGTCAGGGTTTTTCCAGTGAC-3') and T-DNA left border primer Lba1 (5'-TGGTTCACGTAGTGGGCCATCG-3'). The seedlings were grown on MS medium with 1.37% sucrose (except for the Results in Table 1A and 1B, where sucrose was omitted) under cLL; 30 µmol $m^{-2}s^{-1}$, cML; 80 µmol $m^{-2}s^{-1}$ or cHL; 200 µmol $m^{-2}s^{-1}$ at 20°C. Alternatively, growth occurred under SD or LD conditions in combination with the three different light intensities. For long-term experiments (18 days) in plates and root analyses, the seedlings were grown on Hoagland medium in square plates and vertical orientation. For experiments on soil, 14-day old seedlings grown on MS in cLL were transferred to pots containing garden soil and vermiculite (9:1; v/v) and cultivated under the given light regimes at 20°C, as outlined in the text or figure legends. Immediately after budding, tripod aracon tubes were placed on every plant to synchronize growth of the individual plants and to prevent cross pollination. After complete drying of the plants, seeds were harvested and the biomass was determined.

Experiments with *P. indica*

P. indica was cultured on Kaefer medium as described in Johnson et al. (2011). The cocultivation experiment with *P. indica* was done as described in Johnson et al. (2011, Method 2). Control seedlings were transfered to plates inoculated with a plaque without fungal hyphae. Plates were incubated at 20°C under the light regimes described in the text or figure legend. Spores of *P. indica* were harvested from 4-week old fungal plates and resuspended in sterile water at a concentation of 10^6-10^7 ml^{-1}. One ml of spore suspension was added to 1 l of MS or Hoagland media.

Experiments with *A. brassicae*

A. brassicae (FSU-3951, Jena Microbial Resource Center) was grown on PDA medium (pH 6.5) at 22°C, 12/12 h light/dark and 75% relative humidity in a temperature controlled growth chamber. After two weeks, the dense fungal mycelium sporulated heavily. The medium was removed by filtering through 4 layers of sterilized nylon membrane, and the mycelium with spores was washed 3 times with sterile H_2O. The spores and mycelia were gently resuspended in 100 ml of sterile H_2O and filtered through four layers of sterilized nylon membrane to remove the mycelia and hyphae. The spore density was adjusted to 10^5-10^6 ml^{-1}, either by

serial dilution or a Haemocytometer. For leaf infection, 14-day old seedlings were transferred to fresh PNM plates with a sterilized nylon membrane and incubated at 20°C under LD ML conditions (for details see Johnson et al., 2011). After 48 h, 5 µl of the spore suspension was inoculated on to 6 leaves in the middle whorl per seedling and were incubated in a temperature controlled growth chamber as described earlier. After 3-7 days, progression of disease development was quantified as Percentage Disease Index (PDI) using standard disease intensity grades based on the number and area of leaves infected.

The competitive experiments with *P. indica* and *A. brassicae* on Arabidopsis seedlings were performed by cocultivating the roots with *P. indica* and inoculating the leaves with *A. brassicae* spores 48 h later. *A. thaliana* was cocultivated with *P. indica* on modified PNM medium as described in Johnson et al. (2011). After 48 h of cocultivation, the upper 6 leaves are inoculated with 5 µl of *A. brassicae* spore suspension (10^5-10^6 spores ml^{-1}) and incubated as described earlier. The seedlings not treated with *P. indica* but inoculated with *A. brassicae* served as control. The toxin from *A. brassicae* was prepared according to Vidyasekaran et al. (1997). The toxin was further concentrated, lyophilised and resuspended in sterile H_2O.

Plasmid construction and plant transformation

For the *RRTF1* (At4g34410) oe construct p35S-*RRTF1*, the *RRTF1* coding region was amplified from *RRTF1* cDNA clone (DKLAT4G34410), obtained from Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). The PCR product was cloned into pCR®8/GW/TOPO® TA vector (Invitrogen), and then the insert was integrated into a binary vector, pB2GW7 containing CaMV 35S promoter, using Gateway LR recombination reaction system (Invitrogen). p35S-*RRTF1* was transformed into Arabidopsis Col-0 via *Agrobacterium tumefaciens* strain GV3101 with floral-dip method. T1 plants were selected by spraying 1-2 weeks old seedlings with 0.1% BASTA (Bayer, Germany). T2- and T3-plant resistant to DL-phosphinothricin (50 µM) (Sigma-Aldrich) on MS medium plate were used for physiological experiments.

RNA analyses

Total RNA was isolated from shoot and roots with the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from 1 µg total RNA with Omniscript RT Kit (Qiagen) and oligo (dT)20 in 20 µl reaction volume. Semi-quantitative and quantitative real-time PCR were done with gene specific primers (Supplemental Material 12). Real-time quantitative RT-PCR was

performed using the iCycler iQ real-time PCR detection system and iCycler software version 2.2 (Bio-Rad). For the amplification of the PCR products, iQ SYBR Supermix (Bio-Rad) was used according to the manufacturer´s instructions in a final volume of 23 µl. The iCycler was programmed to 95°C 2 min, 35× (95°C 30 s, 55°C 40 s, 72°C 45 s), 72°C 10 min followed by a melting curve programme (55–95°C in increasing steps of 0.5°C). All reactions were repeated twice. The mRNA levels for each cDNA probe were normalized with respect to the *GAPDHC* message levels. Fold induction values were calculated with the ΔΔCP equation of Pfaffl (2001). The ratio of a target gene was calculated in the treated sample versus the untreated control in comparison to a reference gene.

Microarray analysis

Total RNA was isolated from mature leaves on soil under SD ML or cotyledons of seedlings grown on MS under SD LL and the microarray hybridization was performed with the Arabidopsis Genome Array ATH1 (Affymetrix, USA) at the Kompetenz zentrum für Fluoreszente Bioanalytik, Regensburg, Germany. The hybridization signal data were analyzed with ROBIN program (http://mapman.gabipd.org/web/ guest/robinsoftware) and MapMan program (http://mapman.gabipd.org/web/guest/ mapman). Statistical analysis for t-test and subsequent calculation of false discovery rate (FDR) was according to ROBIN program with the data of 3 biological independent experiments.

DCMU treatment

The electron transport inhibitor DCMU (Sigma) was applied to the seedlings in Petri dishes by spraying 0.5 ml of a 5 µM solution to the leaves 90 min before transfer of the seedlings from LL to HL or before the application of the PAMPs. Control seedlings were treated with the solvent without DCMU.

PAMP treatments with flg22, chitin or an *A. brassicae* toxin

Each leaf of 10-day old Arabidopsis seedlings were treated with 20 µl of 1 µM flg22, or 1 µM chitin (crab shells, Sigma-Aldrich) or *A. brassicae* toxin preparation and the control seedlings with 20 µl of sterile H_2O.

Quantitative H_2O_2 and ROS measurements and detection of ROS in roots

Quantitative H_2O_2 measurement from leaves and roots were performed using the Amplex Red hydrogenperoxide/peroxidase assay kit (Molecular Probes) according to the manufacturer's

instructions (http://tools.invitrogen.com/content/sfs/manuals/mp 22188.pdf). Leaf sections of 0.5-1 mm width and root sections of 2-3 cm length were incubated in the reaction mixture for 10 min in dark at room temperature. The fluorescence intensity was quantified with a fluorescence microplate reader (TECAN Infinite 200) with an excitation at 540 nm and emission at 610 nm. H_2O_2 was used to prepare the standard curve. The reaction mixture without the substrate and plant material served as control. ROS measurements from leaves and roots were performed using the substrate carboxy-H_2DFFDA according to the manufacturer's instructions (https://tools.invitrogen.com/content/sfs/ manuals/mp36103.pdf). The plant material was incubated in 20 µM carboxy-H_2DFFDA prepared in KRPG buffer for 30 min in the dark. The fluorescence intensity was quantified with a fluorescence microplate reader (TECAN Infinite 200) with an excitation at 485 nm and emission at 530 nm. The reaction mixture without the substrate and plant material served as control. ROS imaging in roots were performed by fixing their ends on a glass slide and by incubating the root sections with 20 µM carboxy-H_2DFFDA prepared in KRPG buffer for 30 min. The treated roots were washed 5 times with KRPG buffer without mechanical disturbances and immediately photographed using Axiocam MRC5 fluorescence microscope (Carl Zeiss, Germany).

Miscellaneous

For quantification of photobleached leaf areas, the white areas of the leaf were quantified as pixel in Photoshop™ on a photo. Measurement of photosynthesis parameters and analysis of chlorophyll and anthocyanin contents are described in the Supplemental Material 3.

ACKNOWLEDGEMENTS

M.M. was supported by the Alexander-von-Humboldt foundation and J.M.J. by the IMPRS Jena. We like to thank Dr. N.N. Kav (University of Alberta, Canada) for the *RAP2.6* oe lines; Prof. Scheel for providing flg22; and Sarah Mußbach and Claudia Röppischer for their excellent technical assistance.

REFERENCES

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. **55:** 373-399.

Asada K. (2000). The water-water cycle as alternative photon and electron sinks. Philos: Trans: R: Soc: Lond: B Biol. Sci. **355:** 1419-1431.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and

their functions. Plant Physiol. **141:** 391-396.

Balazadeh, S., Jaspert, N., Arif, M., Mueller-Roeber B., and Maurino V. G. (2012). Expression of ROS-responsive genes and transcription factors after metabolic formation of H_2O_2 in chloroplasts. Front. Plant Sci. **3:** 1-30.

Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.H., Schäfer, P., Schwarczinger, I., Zuccaro, A., Skoczowski, A. (2008). Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol. **180:** 501-510.

Böhmer, M., and Schroeder, J. I. (2011). Quantitative transcriptomic analysis of abscisic acid-induced and reactive oxygen species-dependent expression changes and proteomic profiling in Arabidopsis suspension cells. Plant J. **67:** 105-118.

Carol, R.J., and Dolan, L. (2006). The role of reactive oxygen species in cell growth: lessons from root hairs. J. Exp. Bot. **57:** 1829-1834.

Chen, H., Lai, Z., Shi, J., Xiao, Y., Chen, Z., and Xu, X. (2010). Roles of Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. BMC Plant Biol. **10:** 281.

Dietz, K.J., Jacob, S., Oelze, M.L., Laxa, M., Tognetti, V., de Miranda, S.M., Baier, M., and Finkemeier, I. (2006). The function of peroxiredoxins in plant organelle redox metabolism. J. Exp. Bot. **57:** 1697-1709.

Dietz, K.J., Vogel, M.O., and Viehhauser, A. (2010). AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signaling. Protoplasma **245:** 3-14.

Fester, T., and Hause, G. (2005). Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza **15:** 373-379.

Foyer, C.H., and Noctor, G. (2009). Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid. Redox Signal. **11:** 861-905.

Fujita, M., Fujita, Y., Iuchi, S., Yamada, K., Kobayashi, Y., Urano, K., Kobayashi, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2012). Natural variation in a polyamine transporter determines paraquat tolerance in Arabidopsis. Proc. Natl. Acad. Sci. USA. **109:** 6343-6347.

Funck, D., Eckard, S., and Müller, G. (2010). Non-redundant functions of two proline dehydrogenase isoforms in Arabidopsis. BMC Plant Biology **10:** 70. doi:10.1186/1471-2229-10-70.

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inzé, D., Mittler, R., and Van Breusegem, F. (2006). Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. Plant Physiol. **141:** 436-545.

Galvez-Valdivieso, G., and Mullineaux, P.M. (2010). The role of reactive oxygen species in signaling from chloroplasts to the nucleus. Physiol. Plant. **138:** 430-439.

Heitz, T., Widemann, E., Lugan, R., Miesch, L., Ullmann, P., Désaubry, L., Holder, E., Grausem, B., Kandel, S., Miesch, M., Werck-Reichhart, D., and Pinot, F. (2012). Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of

the plant hormone jasmonoyl-isoleucine for catabolic turnover. J. Biol. Chem. **287:** 6296-6306.

Heller, J., and Tudzynski, P. (2011). Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annu. Rev. Phytopathol. **49:** 369-390.

Jaspers, P., and Kangasjärvi, J. (2010). Reactive oxygen species in abiotic stress signaling. Physiol. Plant. **138:** 405-413.

Johnson, J.M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Lou, B., Varma, A., and Oelmüller, R. (2011). Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation – A model system to study plant beneficial traits. Endocyt. Cell Res. **21:** 101-113. (http://zs.thulb.uni-jena.de/receive/jportal_jparticle_00247947).

Khandelwal, A., Elvitigala, T., Ghosh, B., and Quatrano, R.S. (2008). *Arabidopsis* transcriptome reveals control circuits regulating redox homeostasis and the role of an AP2 transcription factor1. Plant Physiol. **148:** 2050-2058.

Kimura, M., Yamamoto, Y.Y., Seki, M., Sakurai, T., Sato, M., Abe, T., Yoshida, S., Manabe, K., Shinozaki, K., and Matsui, M. (2003). Identification of Arabidopsis genes regulated by high light-stress using cDNA microarray. Photochem Photobiol. **77:** 226-233.

Krishnaswamy, S., Verma, S., Rahman, M.H., and Kav, N.N. (2011). Functional characterization of four APETALA2-family genes (RAP2.6, RAP2.6L, DREB19 and DREB26) in Arabidopsis. Plant Mol. Biol. **75:** 107-127.

Laloi, C., Przybyla, D., and Apel, K. (2006). A genetic approach towards elucidating the biological activity of different reactive oxygen species in *Arabidopsis thaliana*. J. Exp. Bot. **57:** 1719-1724.

Lamb, C., and Dixon, R.A. (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48:** 251-275.

Lee, D.J., Park, J.Y., Ku, S.J., Ha Y.M., Kim, S., Kim, M.D., Oh, M.H., and Kim, J. (2007). Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7 (ARR7) overexpression in cytokinin response. Mol. Genet. Genomics **277:** 115-137.

Lejay, L., Wirth, J., Pervent, M., Cross, J.M., Tillard, P., and Gojon A. (2008). Oxidative pentose phosphate pathway-dependent sugar sensing as a mechanism for regulation of root ion transporters by photosynthesis. Plant Physiol. **146:** 2036-2053.

Marino, D., Dunand, C., Puppo, A., and Pauly, N. (2012). A burst of plant NADPH oxidases. Trends Plant Sci. **17:** 9-15.

Matsui, A., Ishida, J., Morosawa, T., Mochizuki, Y., Kaminuma, E., Endo, T.A., Okamoto, M., Nambara, E., Nakajima, M., Kawashima, M., Satou, M., Kim, J.M., Kobayashi, N., Toyoda, T., Shinozaki, K., and Seki, M. (2008). Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant Cell Physiol. **49:** 1135-1149.

Mehterov, N., Balazadeh, S., Hille, J., Toneva, V., Müller-Röber, B., and Gechev, T. (2012). Oxidative stress provokes distinct transcriptional responses in the stress-tolerant *atr7* and stress-sensitive *loh2 Arabidopsis thaliana* mutants as revealed by multi-parallel quantitative real-time PCR analysis of ROS marker and antioxidant genes. Plant Physiol. Biochem. **59:** 20-29.

Mersmann, S., Bourdais, G., Rietz, S., and Robatzek, S. (2010). Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. Plant Physiol. **154:** 391-400.

Miao, Y., and Zentgraf, U.A. (2010). HECT E3 ubiquitin ligase negatively regulates Arabidopsis leaf senescence through degradation of the transcription factor WRKY53. Plant J. **63:** 179-188.

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M.A., Shulaev, V., Dangl, J.L., and Mittler, R. (2009). The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Sci. Signal. **2:** ra45.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., and Mittler, R. (2010). Reactive oxygen species homeostasis and - 129 -signaling during drought and salinity stresses. Plant Cell Environ. **33:** 453-467.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. Trends Plant Sci. **9:** 490-498.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V., and Van Breusegem, F. (2011). ROS signaling: the new wave? Trends Plant Sci. **16:** 300-309.

Mu, R.L., Cao, Y.R., Liu, Y.F., Lei, G., Zou, H.F., Liao, Y., Wang, H.W., Zhang, W.K., Ma, B., Du, J.Z., Yuan, M., Zhang, J.S., and Chen, S.Y. (2009). An R2R3-type transcription factor gene AtMYB59 regulates root growth and cell cycle progression in Arabidopsis. Cell Res. **19:** 1291-1304.

Nakano T, Suzuki, K, Fujimura, T, and Shinshi H. (2006). Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol. **140:** 411-432.

Nakatsubo, T., Mizutani, M., Suzuki, S., Hattori, T., and Umezawa, T. (2008). Characterization of *Arabidopsis thaliana* pinoresinol reductase, a new type of enzyme involved in lignan biosynthesis. J. Biol. Chem. **283:** 15550-15557.

Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W.R., Hahlbrock. K., and Scheel, D. (1994). High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell **78:** 449-460.

Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., Hishinuma, H., Senzaki, E., Yamagoe, S., Nagata, K., Nara, M., Suzuki, K., Tanokura, M. and Kuchitsu, K. (2008). Synergistic activation of the Arabidopsis NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. J. Biol. Chem. **283:** 8885-8892.

Op den Camp, R.G., Przybyla, D., Ochsenbein, C., Laloi, C., Kim, C., Danon, A., Wagner, D., Hideg, E., Göbel, C., Feussner, I., Nater, M., and Apel, K. (2003). Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. Plant Cell 1**5:** 2320-2332.

Pandey, S.P., Roccaro, M., Schön, M., Logemann, E., and Somssich, I.E. (2010). Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. Plant J. **64:** 912-923.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:** e45.

Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H., and Knight, M.R. (2004). OXI1 kinase is necessary for oxidative burst-mediated signaling in Arabidopsis. Nature **427:** 858-861.

Robatzek, S., and Somssich, I.E. (2001). A new member of the Arabidopsis WRKY transcription factor family, AtWRKY6, is associated with both senescence-and defense-related processes. Plant J. **28:** 123-133.

Rossel, J.B., Wilson, P.B., Hussain, D., Woo, N.S., Gordon, M.J., Mewett, O.P., Howell, K.A., Whelan, J., Kazan, K., and Pogson, B.J. (2007). Systemic and intracellular responses to photooxidative stress in Arabidopsis. Plant Cell **19:** 4091-4110.

Rouhier, N. (2010) Plant glutaredoxins: pivotal players in redox biology and iron-sulphur centre assembly. New Phytol. **186:** 365-372.

Saibo, N.J., Lourenço, T., and Oliveira, M.M. (2009). Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Ann. Bot. **103:** 609-623.

Sønderby, I.E., Burow, M., Rowe, H.C., Kliebenstein, D.J., and Halkier, B.A. (2010). A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in Arabidopsis. Plant Physiol. **153:** 348-363.

Swanson, S., and Gilroy, S. (2010). ROS in plant development. Physiol. Plant. **138:** 384-392.

Torres, M.A. (2010). ROS in biotic interactions. Physiol. Plant. **138:** 414-429.

Torres, M.A., and Dangl, J.L. (2005). Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr. Opin. Plant Biol. **8:** 397-403.

Toufighi, K., Brady, S.M., Austin, R., Ly, E., and Provart, N.J. (2005). The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. Plant J. **43:** 153-163.

Tripathy, B., and Oelmüller, R. (2012). Reactive oxygen species generation and signaling in plants. Plant Signal Behav. **7:** 1621-1633.

Vadassery, J., Tripathi, S., Prasad, R., Varma, A., and Oelmüller, R. (2009). Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and Arabidopsis. J Plant Physiol. **166:** 1263-1274.

Vahisalu, T., Puzõrjova, I., Brosché, M., Valk, E., Lepiku, M., Moldau, H., Pechter, P., Wang, Y.S., Lindgren, O., Salojärvi, J., Loog, M., Kangasjärvi, J., and Kollist, H. (2010). Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. Plant J. **62:** 442-453.

Vanderauwera, S., Zimmermann, P., Rombauts, S., Vandenabeele, S., Langebartels. C., Gruissem, W., Inzé, D., and Van Breusegem, F. (2005). Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiol. **139:** 806-821.

Vidhyasekaran, P., Ruby Ponmalar, T., Samiyappan, R., Velazhahan, R.,Vimala, R., Ramanathan, A., Paranidharan, V., and Muthukrishnan, S. (1997). Host-specific toxin production by *Rhizoctonia solani*, the rice sheath blight pathogen. Phytopathol. **87:** 1258-1263.

Wagner, D., Przybyla, D., Op den Camp, R., Kim, C., Landgraf, F., Lee, K.P., Würsch,

M., Laloi, C., Nater, M., Hideg, E., and Apel, K. (2004). The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana.* Science **306:** 1183-1185.

Wang, P., and Song, C.P. (2008). Guard-cell signaling for hydrogen peroxide and abscisic acid. New Phytol. **178:** 703-718.

Wang, Z., Cao, G., Wang, X., Miao, J., Liu, X., Chen, Z., Qu, L.J., and Gu, H. (2008). Identification and characterization of COI1-dependent transcription factor genes involved in JA-mediated response to wounding in Arabidopsis plants. Plant Cell Rep. **27:** 125-135.

Wrzaczek, M., Brosché, M., Salojärvi, J., Kangasjärvi, S., Idänheimo, N., Mersmann, S., Robatzek, S., Karpiński, S., Karpińska, B., and Kangasjärvi, J. (2010). Transcriptional regulation of the CRK/DUF26 group of receptor-like protein kinases by ozone and plant hormones in Arabidopsis. BMC Plant Biol. **10:** 95.

Wu, A., Allu, A.D., Garapati, P., Siddiqui, H., Dortay, H., Zanor, M.I., Asensi-Fabado, MA, Munné-Bosch, S., Antonio, C., Tohge, T., Fernie, A.R., Kaufmann, K., Xue, G.P., Müller-Roeber, B., and Balazadeh, S. (2012). JUNGBRUNNEN1, a reactive oxygen species-Responsive NAC transcription factor, regulates longevity in Arabidopsis. Plant Cell **24:** 482-506.

Xu, X., Chen, C., Fan, B., and Chena, Z. (2006). Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell **18:** 1310-1326.

Yamasaki, K., Kigawa, T., Watanabe, S., Inoue, M., Yamasaki, T., Seki, M., Shinozaki, K., and Yokoyama, S. (2012). Structural basis for sequence-specific DNA recognition by an Arabidopsis WRKY transcription factor. J. Biol. Chem. **287:** 7683-7691.

Yun, B.W., Feechan, A., Yin, M., Saidi, N.B., Le Bihan, T., Yu, M., Moore, J.W., Kang, J.G., Kwon, E., Spoel, S.H., Pallas, J.A., and Loake, G.J. (2011). S-nitrosylation of NADPH oxidase regulates cell death in plant immunity. Nature **478**: 264-268.

Zhu, Q., Zhang, J., Gao, X., Tong, J., Xiao, L., Li, W., and Zhang, H. (2010). The Arabidopsis AP2/ERF transcription factor RAP2.6 participates in ABA, salt and osmotic stresresponses. Gene **457:** 1-12.

4.6 Manuscript VI (in preparation)

A cell wall extract and exudates from *Alternaria brassicae* induce cytosolic calcium elevation and is crucial for the enhanced tolerance to biotic and abiotic stress in *Arabidopsis thaliana*

Joy Michal Johnson, Michael Reichelt, Pyniarlang L. Nongbri, Jyothilakshmi Vadassery, Jonathan Gershenzon and Ralf Oelmüller

Supplementary informations are kept as separate files in the attached CD.

A cell wall extract and exudates from *Alternaria brassicae* induce cytosolic calcium elevation and is crucial for the enhanced tolerance to biotic and abiotic stress in *Arabidopsis thaliana*

Joy Michal Johnson[1], Michael Reichelt[2], Pyniarlang L. Nongbri[1], Jyothilakshmi Vadassery[2], Jonathan Gershenzon[2] and Ralf Oelmüller[1*]

(*) corresponding author; email: b7oera@hotmail.de

[1]Institute of General Botany and Plant Physiology, Friedrich-Schiller-Universität Jena, Dornburger Str. 159, 07743 Jena, Germany; [2]Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Str. 8, D-07745 Jena, Germany.

Key words: abiotic stress, *Alternaria brassicae*, biotic stress, camalexin, cell wall extract, cytosolic calcium elevation, exudate preparations, glucosinolates, phytohormones, toxin

Abbreviations: ABA, Abscisic acid; Ab, *A. brassicae*; $[Ca^{2+}]_{cyt}$, cytosolic calcium; *cycam*, cytosolic calcium mutant; CWE, cell wall extract; GLS, glucosinolates; JA, jasmonic acid; PAMP, pathogen-associated molecular pattern; SA, salicylic acid; EPM, exudate preparation from mycelium; EPS, exudate preparation from germinating spores; WT, wild-type

Running title: *A. brassicae* exudates-induced $[Ca^{2+}]_{cyt}$ elevation confers resistance in Arabidopsis

Abstract

Ca^{2+} is an important second messenger in plants and animals. Here we report that (a) low molecular weight exudate component(s) from *Alternaria brassicae* induce cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation. We isolated an ethane methyl sulfonate (EMS) mutant which does not respond to cell wall extract (CWE) and exudate preparations from mycelium (EPM) and germinating spores (EPS) with regard to $[Ca^{2+}]_{cyt}$ elevation but responded normally to the toxin preparation from *A. brassicae*. The mutant is highly sensitive to *A. brassicae* infections and its toxin preparation, and also to drought and water stress. After exposure to *A. brassicae*, the abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) levels are induced more in mutants than in wild-type (WT). The mutant induced low levels of aliphatic glucosinolates (aGLS) and the bioactive (+)-7-*iso*-jasmonoyl-1-isoleucine conjugate ((+)-7-*iso*-JA-Ile) in

response to *A. brassicae* infection. We propose that the mutated gene product is involved in Ca^{2+}-dependent signaling pathways establishing biotic and abiotic stress tolerance.

INTRODUCTION

Plants have evolved effective mechanisms to perceive, transduce and respond to a wide variety of biotic and abiotic signals by modulating intracellular or cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) levels (c.f. Sanders et al., 2002; Lecourieux et al., 2006; Reddy et al., 2011). Under resting conditions, the $[Ca^{2+}]_{cyt}$ is maintained below 100 nm, 10^4 times less than in the apoplastic fluid and 10^4 to 10^5 times less than in different internal Ca^{2+} store organelles (Hetherington and Brownlee, 2004; Dodd et al., 2010). Ca^{2+} is the most tightly regulated ion within cellular compartments, and tight spatial and temporal control of its concentration makes it a versatile signaling molecule in plants (Clapham, 2007; Kudla et al., 2010). Ca^{2+} signaling is composed of a receptor, a system for generating the transient increase in $[Ca^{2+}]_{cyt}$ through Ca^{2+}-pumps and -channels in response to a stimulus, recognition of the specific Ca^{2+}-signature by sensor proteins, transduction of the information to targets and cellular systems responsible for returning $[Ca^{2+}]_{cyt}$ to its pre-stimulus level (Hetherington and Brownlee, 2004; Reddy and Reddy, 2004). In plants, increase in $[Ca^{2+}]_{cyt}$ arises from the influx of Ca^{2+} from the apoplast and/or from the internal stores through specific channels like cyclic nucleotide gated channels (CNGC), glutamate receptor channels (GLR) or two pore Ca^{2+} channels (TPC) (Sanders et al., 2002; Hetherington and Brownlee, 2004). H^+/Ca^{2+} antiporters and Ca^{2+}-ATPases pump the Ca^{2+} ions back into the apoplast and/or intracellular stores once the receptor is no longer activated by ligand binding (Hetherington and Brownlee, 2004; Reddy and Reddy, 2004).

$[Ca^{2+}]_{cyt}$ elevation is one of the earliest physiological events in root and leaf cells in response to pathogenic stimuli. Upon perception of signals from pathogenic fungi or/and its pathogen-associated molecular patterns (PAMPs), $[Ca^{2+}]_{cyt}$ levels transiently increase in the host cells within seconds (Blume et al., 2000; Müller et al., 2000; Lecourieux et al., 2002, 2005; Hu et al., 2009; Ranf et al., 2012). Plants discriminate both the nature and strength of these stimuli to mount an appropriate rapid adaptive response for their survival (Mithöfer and Mazars, 2002). The interplay of Ca^{2+} and its information processing during plant-fungus interactions is the key determinant in the decision either to establish disease resistance or susceptibility (Lecourieux et al., 2006; Dodd et al., 2010; Reddy et al., 2011).

Recognition and perception of fungal pathogens and their PAMPs induce $[Ca^{2+}]_{cyt}$

elevation which leads to the activation of defence-signaling cascades against the attempted pathogen invasion (Blume et al., 2000; Klüsener et al., 2002). High-affinity binding of PAMPs to pattern recognition receptors activates innate immune responses that trigger basal resistance or PAMP-triggered immunity (PTI) (Nürnberger et al., 1994; Bourque et al., 1999). However, during the coevolution with host plants, fungal pathogens acquired effector molecules e.g. host specific toxins, to suppress PTI. The plants counteract this with a second level of immunity and specific resistance proteins, resulting in effector-triggered immunity (ETI) or classical gene-for-gene resistance (Jones and Dangl, 2006; Boller and Felix, 2009; Millet et al., 2010). Downstream of PAMP-receptor and effector-receptor interactions is a chain of events leading to defense-related gene activation consisting of the regulation of protein kinases and phosphatases, phospholipases, NADPH oxidases and ion channels, and the production of various metabolites such as reactive oxygen species (ROS), salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and phytoalexins (Lecourieux et al., 2006; Boller and Felix, 2009; Millet et al., 2010). Pharmacological studies with inhibitors of $[Ca^{2+}]_{cyt}$ elevation prevented the activation of defense responses (Blume et al., 2000; Lecourieux et al., 2002; Hu et al., 2009), which demonstrates again the important role of Ca^{2+} in plant immunity.

Alternaria brassicae (Berk.) Sacc. (*Ab*) is a necrotrophic deuteromycete fungus which causes black spot disease in crucifers including *A. thaliana*. It is a seed-, air- and soil-borne fungus that penetrates through all plant parts and causes lesions on leaves, stems and siliques (Bains and Tewari, 1987). The disease progression ultimately results in plant death, mostly caused by host-specific toxins (HSTs) (Walton, 1996; Moebius and Hertweck, 2009; Pedras and Khallaf, 2012). HSTs are low molecular weight secondary metabolites of different chemical classes which can be isolated from liquid cultures or germinating spores. The two well-known phytotoxins destruxin B and sirodesmin PL from *A. brassicae* induce phytoalexin and camalexin biosynthesis in crucifers (Pedras and Khallaf, 2012). Here we show that low molecular weight components from a cell wall extract (CWE), from exudates preparations (EP) from the mycelium (EPM) and germinating spores (EPS), and a toxin preparation of *A. brassicae* induce $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis roots stably expressing the Ca^{2+} reporter protein aequorin. We have isolated and characterized a *cytosolic calcium elevation mutant* (*ab-cycam*) which does not induce $[Ca^{2+}]_{cyt}$ elevation in response to CWE, EPM and EPS from *A. brassicae*, but responds to the toxin. *Ab-CYCAM* mutant is highly susceptible to *A. brassicae* infection, sensitive to its toxin preparation and impaired in defense.

RESULTS

Exudate preparations from *A. brassicae* induce $[Ca^{2+}]_{cyt}$ elevation in *Arabidopsis* roots

Under resting conditions, 18-day old transgenic *A. thaliana* (pMAQ2) roots gave $[Ca^{2+}]_{cyt}$ values of 70 ± 0,6 nM (n = 16). After the application of a CWE/EPM/EPS/ a toxin preparation from *A. brassicae* to the roots, a transient increase in the $[Ca^{2+}]_{cyt}$ concentration is observed (Fig. 1A-D). Discharge at the end of the experiment demonstrates that less than 5 of the reconstituted aequorin was consumed after the stimuli, which ensures that the.. amount of aequorin in the sample is not limiting for the Ca^{2+} signal (data not shown, Mithöfer.. and Mazars, 2002). After a lag phase of 15 - 20 s, the levels of $[Ca^{2+}]_{cyt}$ begin to rise and reach a peak of ~ 300 - 400 nM after 40 to 45 s (Fig. 1A-D). Subsequently the Ca^{2+} levels steadily decreased. No $[Ca^{2+}]_{cyt}$ elevation is observed with the water control treatment (Fig. 1A-D) and barely any $[Ca^{2+}]_{cyt}$ elevation is observed in response to the CWE and EPs in the cotyledons of 18-day old seedlings, while the toxin preparation induces $[Ca^{2+}]_{cyt}$ elevation in the cotyledons although at lower rates than in the roots (Fig. 1A-D, insets). For all stimuli, the magnitudes of the $[Ca^{2+}]_{cyt}$ responses are dose-dependent (Suppl. Fig. 1). The *A. brassicae*- CWE, EPM, EPS and the toxin preparation showed very similar $[Ca^{2+}]_{cyt}$ elevation kinetics which did not change after heat treatment (20 min at 121°C by autoclaving) indicating that the components are thermostable (Suppl. Fig. 2). After ethyl acetate extraction most of the activities remained

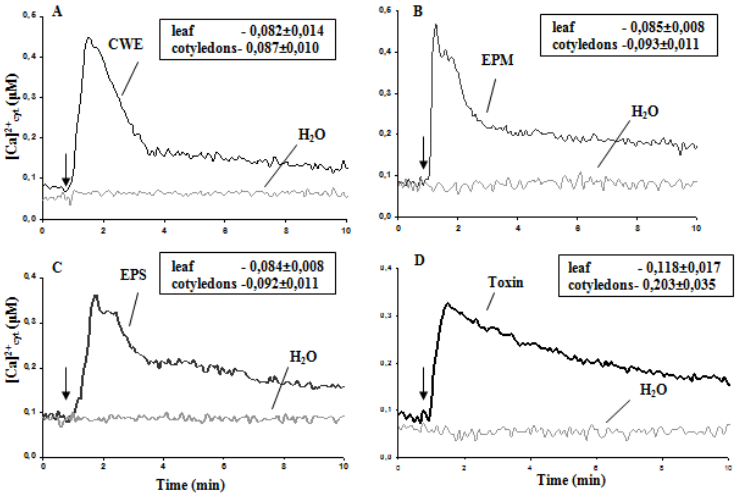


Fig. 1. *A. brassicae*-CWE, -EPM, -EPS and -toxin induces $[Ca^{2+}]_{cyt}$ elevation in *A. thaliana* seedlings expressing cytosolic aequorin. Roots of 18-day old seedlings were challenged with 50 µl of the CWE, EPM, EPS or toxin preparation. $[Ca^{2+}]_{cyt}$ level was calculated from the relative light unit (RLU) at 5 second (s) integration time for 10 min. The arrow indicates the time (1 min) of addition of the stimuli/water. The inset shows the peak values of $[Ca^{2+}]_{cyt}$ elevation in leaves and cotyledons with the same dose of stimuli. In all the experiments, sterile water was used as control and gave background readings. All curves and values (mean ± SE) are from five independent experiments with eight replications in each experiment.

in the aqueous phase. Similar results were obtained after extracting the CWE and EPs preparations with methanol, whereas extraction of the toxin preparation with methanol resulted in a supernatant and precipitate fraction which showed $[Ca^{2+}]_{cyt}$ inducing activities (Suppl. Fig. 2). This suggests that the $[Ca^{2+}]_{cyt}$ activity induced by the toxin preparation is different from those induced by the three extracts. Size separation of the fungal components demonstrates that all compounds are < 3 kD (Suppl. Fig. 2).

A Ca^{2+}-based screen to isolate mutants defective in $[Ca^{2+}]_{cyt}$ elevation to the CWE

White 96-well plates in combination with a plate-reader luminometer equipped with an automatic injection system was used to screen for Arabidopsis mutants which do not show $[Ca^{2+}]_{cyt}$ elevation in response to the CWE. The screen was performed with roots of individual 18-day old M_2 seedlings grown on Hoagland (HL) medium, from EMS mutagenised-M_1 seeds in the Col-0 background (pMAQ2; Knight et al., 1991, 1997). After recording the background $[Ca^{2+}]_{cyt}$ level for 1 min, the response to the CWE was measured for 10 min. Roots which did not respond to the stimulus were used for the discharge reaction to ensure that the lack of $[Ca^{2+}]_{cyt}$ elevation is not caused by a mutation in the aequorin gene. Screening of approximately 150.000 individual M_2 plants identified 12 mutants which failed to induce $[Ca^{2+}]_{cyt}$ elevation in response to the CWE (Fig. 2A), and were named as *cytoplasmic calcium* (cyca) *mutants* to *A. brassicae*-CWE (*ab-cycam*). The putative mutants were transferred to soil and allowed to self to obtain M_3 (up to M_6) seeds. Three putative mutants did not survive in soil. For the other lines, the phenotype was confirmed with the M_3 and M_4 material. None of them show a visible phenotype under our growth conditions when compared to wild-type (WT) (Fig. 2B). Genetic analyses of crosses uncovered that four *Ab-CYCAM* mutants were

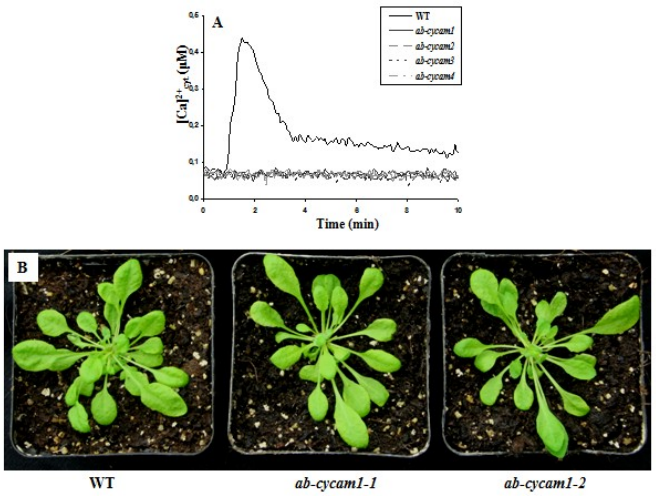


Fig. 2. Screening for mutants which do not respond to Ab-CWE. M_2 seedlings from the individual M_1 plants were used for the mutant screening. About 70% of the roots from the individual M_2 seedlings was dissected and incubated in 7.5 µM coelenterazine overnight and challenged with CWE. *ab-cycam1*, *ab-cycam2*, *ab-cycam3* and *ab-cycam4* did not respond to the CWE **(A)**. pMAQ2 in Col-0 served as control. Three weeks old homozygous mutants in soil-vermiculite mix (9:1) grown under short day (SD) (8h/16h - light/Dark) condition at 22°C with a light intensity of 100 µmol photons m^{-2} s^{-1} did not show any pleiotropic effect **(B)**.

allelic. Two of them, *ab-cycam1-1* and *ab-cycam1-2*, were used for further analyses. When *ab-cycam1-1* and *ab-cycam1-2* were backcrossed to WT (Col-0) or WT (La-*er*), $[Ca^{2+}]_{cyt}$ elevation to the CWE was restored in ~25 of F_2 progenies, indicating that the mutations are recessive.

Ab-CYCAM1 mutants do not respond to the CWE and the EPs, but responded to the toxin preparation

When the response of *ab-cycam1* to the four Ca^{2+} inducing stimuli was tested, the roots did not respond to the CWE and EPs, but showed a WT response to the toxin preparation (Fig. 3A-D). To test whether the $[Ca^{2+}]_{cyt}$ responses induced by the four stimuli show a refractory behaviour, roots of WT and *ab-cycam1* were challenged first with either the CWE, EPM or

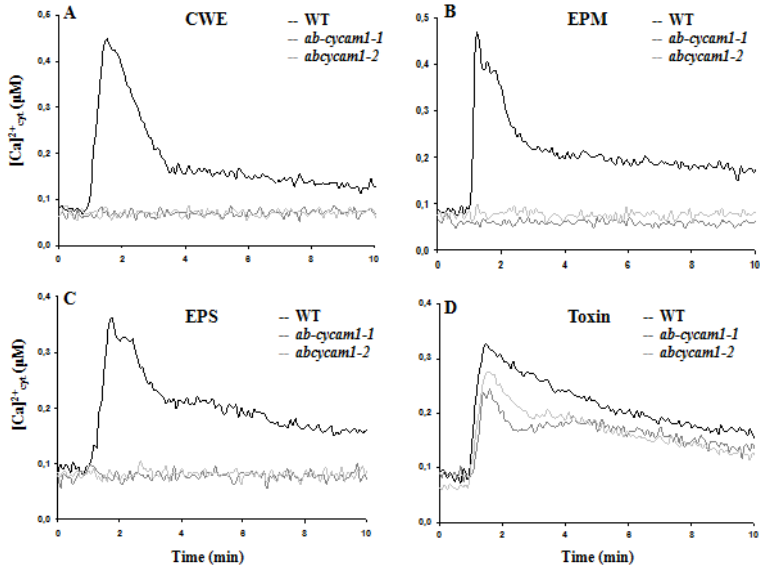


Fig. 3. The response of *ab-cycam1* to different *A. brassicae*-derived stimuli. The roots of both *ab-cycam1-1* and *ab-cycam1-2* were challenged with 50 µl of CWE **(A)**, EPM **(B)**, EPS **(C)** or toxin **(D)**. The mutants did not respond to the CWE and EPs but responded to the toxin preparation. All curves represent mean of four independent experiments with eight replications in each experiment.

EPS and subsequently with either the same stimulus or one of the other two stimuli. Ten min after the first stimulus, when the $[Ca^{2+}]_{cyt}$ level is on its descent, the second stimulus was applied. Fig. 4A-D demonstrate that the second stimulus shows always a weak response, irrespective of whether the CWE or the EPs were used. Since any combination of these stimuli gave comparable $[Ca^{2+}]_{cyt}$ responses with a refractory feature, the *ab-cycam1* mutant is involved in the response to all three stimuli. Therefore, the three preparations contain either the same compound or all of them require the mutated gene for $[Ca^{2+}]_{cyt}$ elevation (*Ab-CYCAM1*) in Arabidopsis roots. If the toxin preparation is applied as a second stimulus, a strong $[Ca^{2+}]_{cyt}$ elevation without refractory feature is observed in WT roots, irrespective of the first stimulus as CWE or EPM or EPS (Fig. 4A-D). Therefore, the toxin preparation-

induced $[Ca^{2+}]_{cyt}$ response is independent of Ab-CYCAM1. Finally, we used flg22 to stimulate $[Ca^{2+}]_{cyt}$ elevation in the leaves and roots of *ab-cycam1* and no difference to the WT was observed (data not shown).

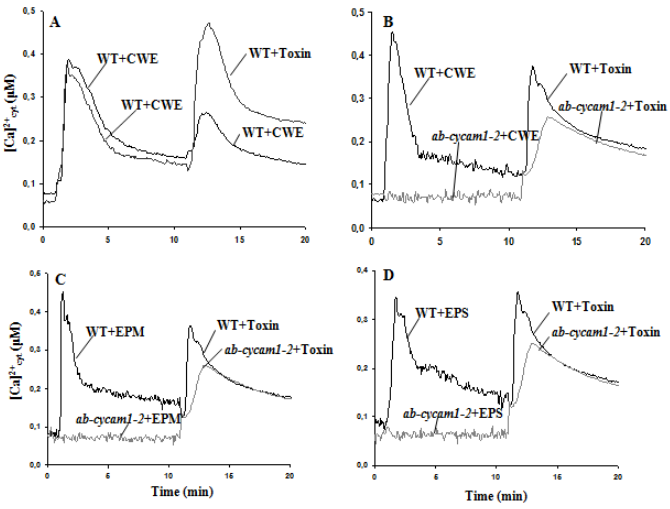


Fig. 4. Refractory behaviour of the fungal stimuli to $[Ca^{2+}]_{cyt}$ changes in WT and *ab-cycam1-2* in competition assays. WT and mutant roots were first treated with either the CWE, EPM or EPS and subsequently 10 min later with CWE or the toxin. The CWE-induced $[Ca^{2+}]_{cyt}$ change is refractory to consecutive applications of CWE but non-refractory to the second treatment with toxin in WT roots **(A)**. The WT roots treated first with the CWE, the EPM or EPS induce $[Ca^{2+}]_{cyt}$ changes, and not in *ab-cycam1-2*, but both WT and mutant responded to subsequent treatment with toxin **(B, C and D)**. All curves represent mean of three independent experiments with eight replications in each experiment.

$[Ca^{2+}]_{cyt}$ elevation is mobilised by the extra- and intra-cellular pools of Ca^{2+}

Arabidiopsis lines with the pMAQ2 construct (Knight et al., 1991, 1997) accumulate aequorin in the cytoplasm and is often used to monitor $[Ca^{2+}]_{cyt}$ elevation due to opening of plasmamembrane-localized Ca^{2+} channels and uptake of Ca^{2+} from external and internal stores. To elucidate the origin of Ca^{2+} flux, we performed pharmacological studies using different chelators/inhibitors which can prevent internal and external mobilisation of Ca^{2+} to cytoplasm. Initially, we determined the maximum concentration of these chelators/inhibitors at which the basal level of $[Ca^{2+}]_{cyt}$ is not changed significantly and also do not affect the discharging of excess reconstituted aequorin with 100mM $CaCl_2$ when the roots are incubated with them for 1 h. Incubation of roots with 5µM staurosporine (a protein kinase inhibitor) 1 h prior to the treatment of CWE, EPM, EPS and toxin completely blocked the $[Ca^{2+}]_{cyt}$ elevation which indicate that *Ab-CYCAM1* has kinase domain(s) (Fig. 5A). 1 mM BAPTA (an external Ca^{2+} chelator blocking the influx of Ca^{2+} from apoplast to cytoplasm through plasma membrane) and 20 µM neomycin, (a phospholipase C (PLC) inhibitor blocking the inositol 1,4,5-triphosphate (IP_3)-mediated Ca^{2+} release from the internal stores) drastically suppressed CWE, EPM, EPS and toxin-induced $[Ca^{2+}]_{cyt}$ elevation (Fig. 5A). In *ab-cycam1*, as shown in the figure 5B, staurosporine treatment completely- whereas BAPTA and neomycin treatments partially inhibited toxin-induced $[Ca^{2+}]_{cyt}$ elevation. These results indicate that $[Ca^{2+}]_{cyt}$

elevations induced by all four stimuli are contributed by both extra- and intra-cellular Ca^{2+} (Fig. 5A-B). We also tested the sensitivity of *ab-cycam1* and WT to Ca^{2+} inhibitors and found that the performance of *ab-cycam1* was severely impaired compared to WT (Suppl. Fig. 3).

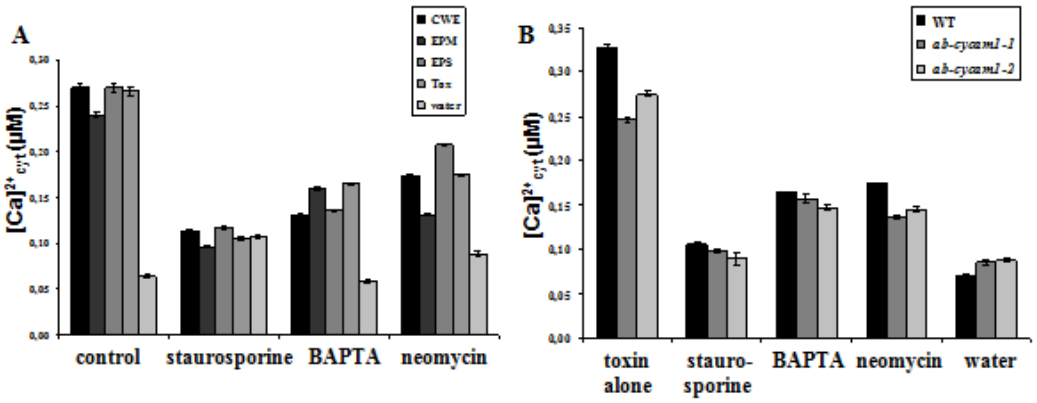


Fig. 5. Extra- and intra-cellular Ca^{2+} are involved in CWE-, EPM-, EPS- and toxin-induced $[Ca^{2+}]_{cyt}$ elevation. Roots of 18-day old seedlings grown on HL medium under long day (LD) condition were dissected and incubated overnight in 7.5 µM coelenterezine. The roots were incubated with different inhibitors/chelators for 1 h after removing the coelenterazine solution without disturbing the roots. The roots were gently washed with sterile water and challenged with 50 µl of different *A. brassicae* stimuli. The effects of different inhibitors/chelators on CWE-, EPM-, EPS- and toxin-induced peak values of $[Ca^{2+}]_{cyt}$ elevation in WT **(A)**; and the toxin-induced peak values of $[Ca^{2+}]_{cyt}$ elevation in *ab-cycam1* **(B)** are shown. The control measurements are without inhibitors/chelators. Mock control treatment was done with sterile water or 0.1 DMSO. Staurosporine - 5 µM, bis(o-amino- phenoxy)ethane-tetraacetic acid (BAPTA) - 1 mM and neomycin - 20 µM were used. All values represent mean ± SE of three independent experiments with sixteen replications in each experiment.

ab-cycam1 plants are highly susceptible to *A. brassicae* infection, the toxin preparation and drought-related stress

Since the *ab-cycam1* were obtained by screening the EMS mutated pMAQ2 line with the *A. brassicae*-CWE, we tested whether *ab-cycam1* is more susceptible to *A. brassicae* root and leaf infections than WT. 14-day old seedlings (roots and cotyledons) or leaves from 4 week old plants were infected with *A. brassicae*. Infection of the roots of Arabidopsis seedlings was performed by exposing them to a 5 mm fungal plug (cf. Material and Methods, Fig. 6A). The leaves of the seedlings (Fig. 6B) or adult plants (Fig. 6E) were infected with 5 µl of a spore suspension. The disease progression measured as percentage disease index (PDI) was determined 3, 5, 7 and 10 days after inoculation (dai) of the seedling leaves (Fig. 6C). Two . allelic mutants of *ab-cycam1* were more sensitive to *A. brassicae* infection than the WT. The higher transcript level of the *A. brassicae* marker *Atr1* gene in the leaves of the *ab-cycam1* seedlings indicates that the mutant cannot efficiently restrict fungal growth (Fig. 6D). This result indicates that Ab-CYCAM1 participates in establishing resistance against *A. brassicae*

infection. Fig. 6F demonstrates the same results, if *A. brassicae* was replaced by the toxin preparation. Thus, Ab-CYCAM1 participates in establishing resistance to the fungus and its toxin preparation. This can also be demonstrated by growing WT and *ab-cycam1* seedlings on

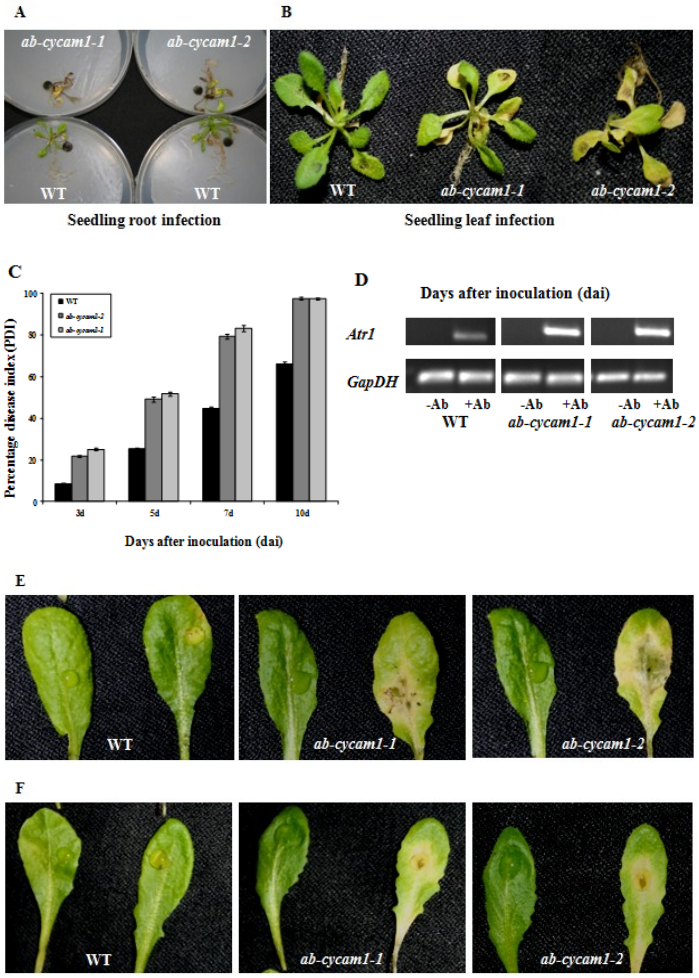


Fig. 6. *ab-cycam1-1* and *ab-cycam1-2* are highly susceptible to *A. brassicae* and its toxin. (A) The roots of 14-day old *ab-cycam1* and WT seedlings were exposed to a fungal plug and incubated under LD conditions for 7 days. **(B)** The leaves of 14-day old *ab-cycam1* and WT seedlings were inoculated with 5 µl spore suspension containing 10^4 - 10^5 cfu ml^{-1} and incubated for 10 days under LD conditions. **(C)** The PDI was determined at 3, 5, 7 and 10 dai of leaves as shown in panel. The values are mean ± SE of 4 independent experiments with twenty four seedlings each. *A. brassicae ATR1* transcript levels are higher in *ab-cycam1-1* and *ab-cycam1-2* leaves than in WT leaves at 5 dai **(D)**. Detached leaf assays with fungal spores **(E)** and toxin **(F).** Mature leaves from 4-week old *ab-cycam1* and WT plant grown under SD conditions were dissected, inoculated with 10 µl spore suspension containing 10^4 - 10^5 cfu ml^{-1} or 10 µl toxin preparation and incubated under LD condition for 5 days. The mock treatment was performed with sterile water. The experiments were repeated four times with twenty four seedlings or leaves, and representative pictures are shown.

media containing low concentrations of the toxin preparation (Fig. 7A-C). False colour images of the seedlings representing Fs/Fm values confirm that WT seedlings suffer barely under the applied low concentration of the toxin preparation while the two *ab-cycam1* mutants do (Fig. 7B). Furthermore, the photosynthetic parameters ´quantum yield of photosystem II´ (ϕPSII), ´photochemical´ (qP) and ´non-photochemical quenching´ (NPQ), and ´maximum quantum yield of PSII´ (Fv/Fm) of the mutants are decreased compared to the WT seedlings exposed to the toxin preparation (Fig. 7B). Thus, the efficiency of the photosynthetic electron transport (ϕPSII, qP), the ability of heat dissipation of photochemical energy (NPQ) and the ratio of functional PSII to total PSII (Fv/Fm) are impaired in the chloroplasts of the mutant plants exposed to the toxin preparation. Taken together, Ab-CYCAM1 participates in

establishing resistance against *A. brassicae* and its toxin preparation.

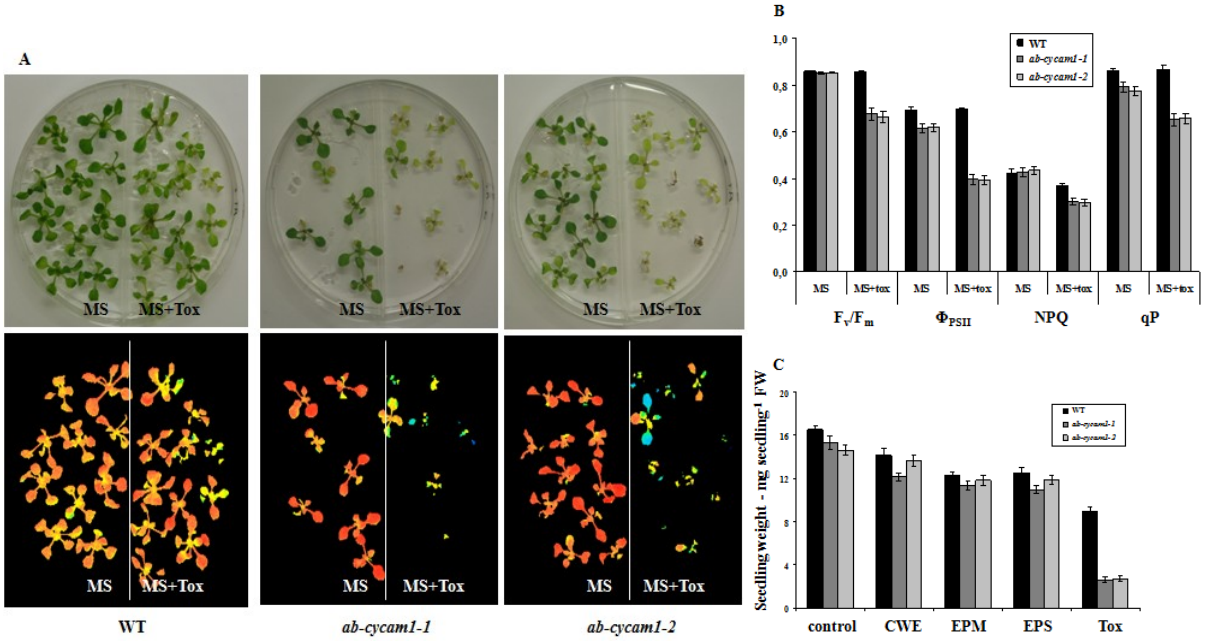


Fig. 7. Response of *ab-cycam1-1* and *ab-cycam1-2* seedlings to CWE, EPM, EPS and the toxin preparation from *A. brassicae*. Mutants and WT seedlings were grown on MS medium supplemented with 3% (v/v) of the different *A. brassicae* stimuli for 14 days and fresh weight (FW) were determined **(A and C)**. The performance of mutants was lower than that of WT. False color pictures representing chlorophyll fluorescence (Fs/Fm values) of (A), where blue represent low and red high F_s/F_m values. Maximum quantum yield of PSII (Fv/Fm), quantum yield of PSII (Φ_{PSII}), photochemical quenching (qP) and non-photochemical quenching (NPQ) were determined from 14-day old WT and mutants seedlings **(B)**. Data are means ± SEs from 6 independent experiments with n>70 seedlings per treatment per line in each experiment. Representative pictures are shown.

Furthermore, when WT and mutant seedlings were grown on MS medium supplemented with either 100 nM ABA or 350 mM mannitol, the fresh weights of the *ab-cycam1* seedlings was significantly reduced compared to the WT (Fig. 8). This suggests that Ab-CYCAM1 plays a role in biotic (*A. brassicae*) and abiotic (water and drought) stress.

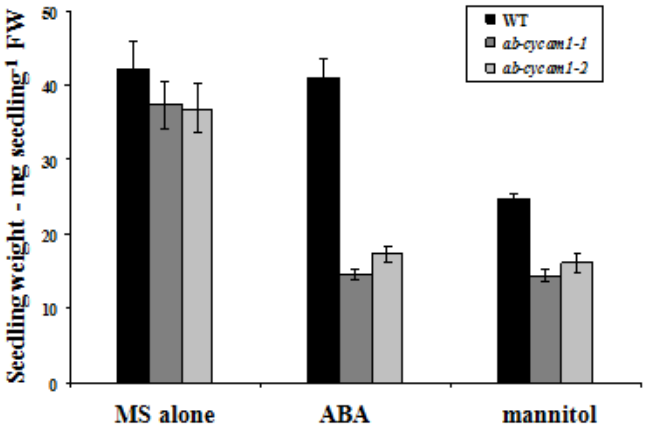


Fig. 8. *ab-cycam1-1* and *ca-cycam1-2* seedlings are sensitive to ABA and mannitol treatments. Mutants and WT seedlings were grown on MS medium supplemented with ABA and mannitol for 20 days before their FW were determined. The values are mean ± SEs of four independent experiments with 40 seedlings of each line per treatement in each experiment.

Phytohormone levels are altered in *ab-cycam1*

SA-, JA- and ABA- (abscisic acid) dependent stress responses are regulated by $[Ca^{2+}]_{cyt}$ levels in plants (Larkindale and Knight 2002; Hu et al. 2009; Vadassery et al. 2012). Stress-related phytohromone levels were determined in the leaves of 14-day old *ab-cycam1* and WT seedlings, with and without exposure to *A. brassicae* spore infection for 3 days. The ABA, SA and JA levels were higher in the mutants compared to WT (Fig. 9A-C), while the JA precursor cis-OPDA (cis-12-oxo-phytodienoic acid) levels were not significantly altered due to *A. brassicae* infection in different lines (Suppl. Fig 4). Interestingly, the biologically inactive JA and (-)-JA-Ile (jasmonic acid-isoleucine conjugate) levels were slightly higher (Fig. 9C and Suppl. Fig. 4), whereas the biologically active form, (+)-7-*iso*-jasmonoyl-1-isoleucine ((+)-7-*iso*-JA-Ile) was significantly low, in *ab-cycam1* compared to WT (Fig. 9D). Therefore, *A. brassicae* infection stimulated SA, ABA, JA and (-)-JA-Ile levels in all plants, but the stimulatory effect was higher for the mutants when compared to the WT. However, the biologically active (+)-7-*iso*-JA-Ile level was significantly high in WT.

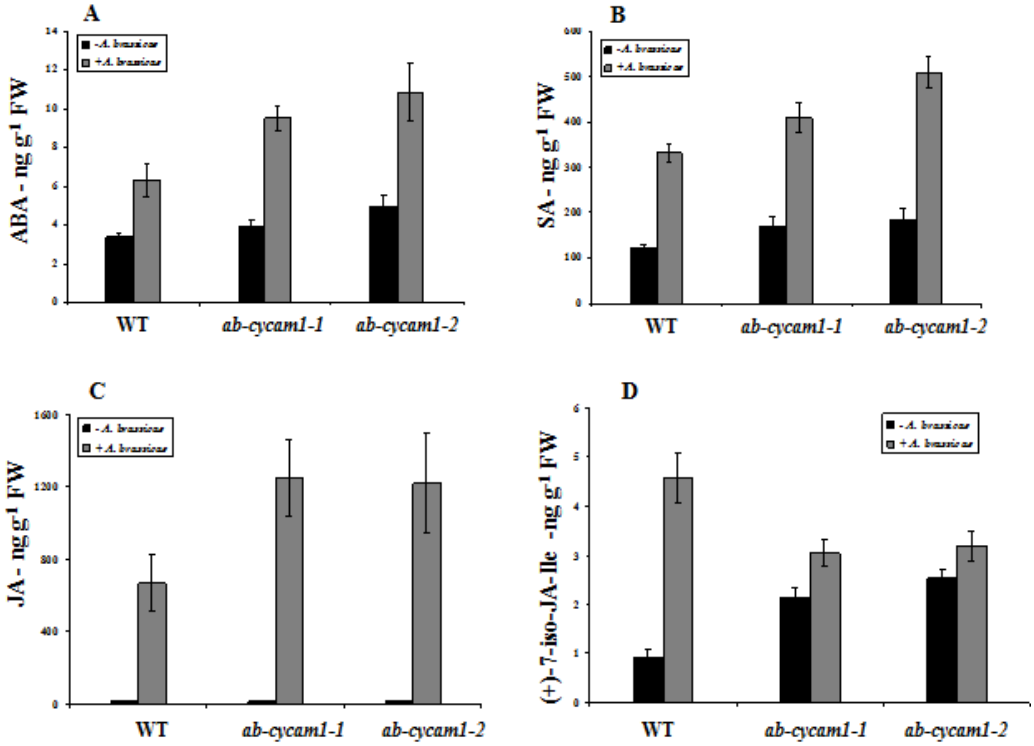


Fig. 9. ABA, SA, JA and (+)-7-*iso*-JA-Ile levels in the leaves of *ab-cycam1* and WT seedlings inoculated with *A. brassicae*. Leaves of 14-day old seedlings grown on MS medium under LD conditions and 80 µmol photons m^{-2} s^{-1} at 22°C were inoculated with *A. brassicae* spore suspension containing 10^4 to 10^5 cfu ml^{-1} or water (control) as described in Methods. ABA **(A)**, SA **(B)**, JA **(C)** and the biologically active form of JA, (+)-7-*iso*-JA-Ile **(D)** levels are shown from the leaves at 3 dai. The values are mean ± SE of four independent experiments with five replications in each experiment.

Ab-CYCAM1 modulates aliphatic glucosinolate levels

In Arabidopsis, camalexin and glucosinolates are the major sulphur containing secondary metabolites which are actively involved in plant defense (Brader et al. 2006; Halkier and Gershenzon 2006; Bednarek et al. 2009). The fungal infection induced both camalexin and indolic glucosinolates (iGLS) production in mutants and WT, however the induction was higher in *ab-cycam1* (Fig. 10A and Suppl. Fig. 5). Three dai with *A. brassicae* spores on leaves, the induction of aliphatic glucosinolates (aGLS) e.g. 5MSOP (5-methylsulfinylpentyl-GLS) and 7MSOH (7-methylsulfinylheptyl-GLS) were significantly higher in WT compared to *ab-cycam1* (Fig. 10B). The expression of *MYB28*, *MYB29* and *BCAT4* which are involved in the aGLS biosynthesis (Halkier and Gershenzon 2006) were also upregulated in WT and not in mutants due to *A. brassicae* infection (Fig. 10C) shows that Ab-CYCAM1 participates in aGLS biosynthesis and thereby defense against *A. brassicae* infection.

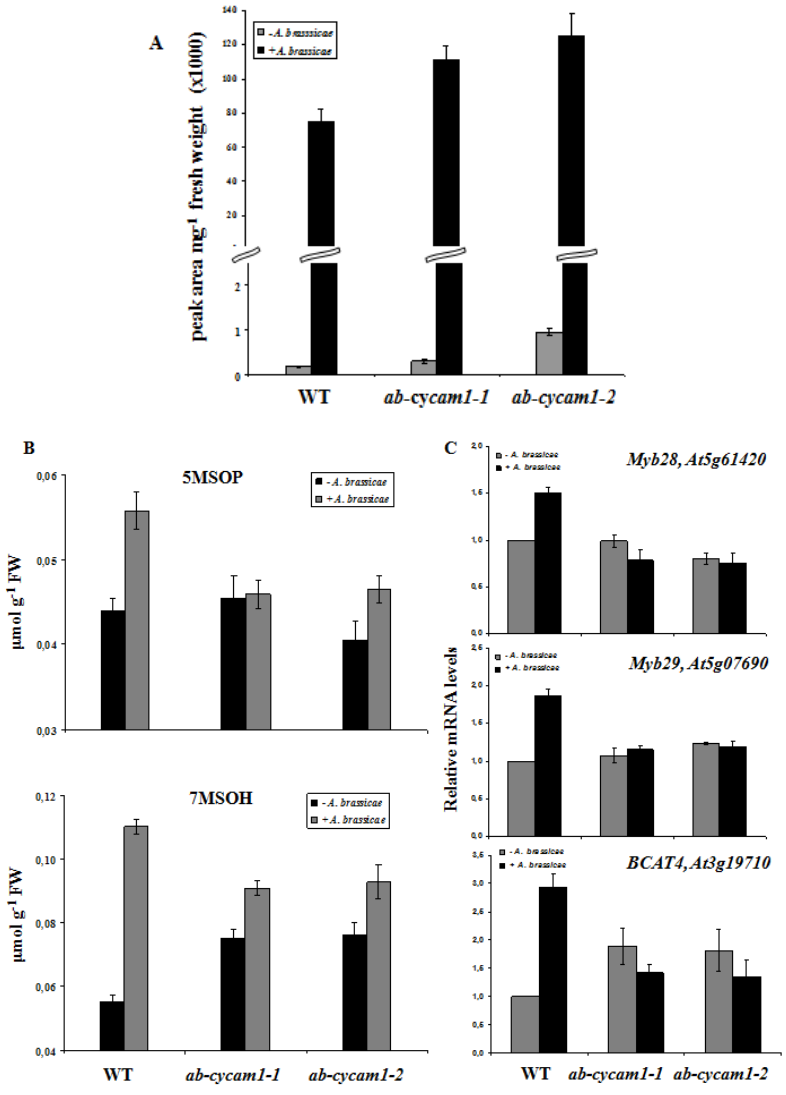


Fig. 10. Induction of camalexin and glucosinolates in *ab-cycam1* in response to *A. brassicae* infection. The leaves of 14-day old seedlings of *ab-cycam1* and WT were inoculated with fungal spore suspension containing 10^4 to 10^5 cfu ml^{-1} and incubated under LD condition. The inoculated seedlings were harvested at 3 dai and used for the measurement of camalexin **(A)**, aliphatic glucosinolates 5MSOP and 7MSOH **(B)** by LC-MS/MS method described in Methods. The values are mean ± SE of 4 independent experiments with 5 replications in each experiment. The relative mRNA levels of aliphatic glucosinolates biosynthesis genes **(C)** from the leaves of different lines at 3 dai of *A. brassicae* are also shown. Mock treatment was done with sterile water. The values are mean ± SE of three independent experiments.

DISCUSSION

A. brassicae exudate compounds trigger $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis roots

A. thaliana plants stably expressing the bioluminescent Ca^{2+} binding aequorin in the cytosol were used to elucidate the role of $[Ca^{2+}]_{cyt}$ changes in the pathogenic interaction with *A. brassicae*. We report that exudate preparations from *A. brassicae* induce $[Ca^{2+}]_{cyt}$ elevation in root cells which in turn modulates defense responses. The response pattern resembles that reported for the identified PAMPs in various plant species, for example, β-glucan from *Phytophthora sojae* in soybean- (Mithöfer et al*.,* 1999), pep-13 from *P. sojae* in parsley- (Blume et al*.*, 2000) and cryptogein from *P. cryptogea* and oligosaccharides in tobacco- (Lecourieux et al., 2002, 2006) cell cultures, pep-25 from *P. sojae* in Arabidopsis (Hu et al., 2009), INF1 from *P. infestans* and boehmerin from *P. boehmeriae* in tobacco (Zhang et al., 2009), chitosan in Arabidospsis (Klüsener et al., 2002), flg22 and elf18 from the flagellated bacteria in Arabidopsis seedlings (Jeworutski et. al., 2010; Ranf et al., 2012). Nod factor from rhizobia (Müller et al., 2000), Myc factor from arbuscular mycorrhiza fungus (Navazio et al., 2007) and Pi-CWE (Vadassery et al., 2009) induce $[Ca^{2+}]_{cyt}$ elevation in roots. The similarity of Ca^{2+} signatures - the lag phase, their dose-dependency, transient appearance and refractory nature suggest that the *A. brassicae* exudate preparations contain (a) compound(s) that may be perceived by a receptor in Arabidopsis root cells (Fig. 1A-D and Suppl. Fig. 1). This is further supported by the inhibition of $[Ca^{2+}]_{cyt}$ elevation by the phosphorylation inhibitor staurosporine. To avoid cytotoxicity, we determined the optimum dose at which the basal level of $[Ca^{2+}]_{cyt}$ and the discharge are not changed. In these conditions, 5 µM staurosporine completely inhibited $[Ca^{2+}]_{cyt}$ elevation in response to the *A. brassicae* preparations (Fig. 5A-B), similar to studies with the cryptogein in tobacco- (Lecourieux et al., 2002), Myc factor in soybean-suspension cultures (Navazio et al., 2007), Pi-CWE in Arabidopsis root (Vadassery et al., 2009) and ergosterol in tobacco (Vatsa et al., 2011). Like flg22 and the Myc factor (Meindl et al., 2000; Navazio et al., 2007), the active components in *A. brassicae* exudate preparations are thermostable, hydrophilic, polar and of low molecular weights (Suppl. Fig. 2).

Isolation and characterization of a *Ab-CYCAM1* mutant

We isolated a loss-of-function mutant, named *ab-cycam1*, which does not show $[Ca^{2+}]_{cyt}$ elevation to CWE, EPM and EPS of *A. brassicae* (Fig. 3A-D). Interestingly, the mutant is also insensitive to CWEs and exudate preparations from the mycelia of necrotrophic fungus

Rhizoctonia solani (FSU 1137) and the hemibiotrophic oomycete fungus *Phytophthora parasitica* var. *nicotianae* (FSU 746) while the Ca^{2+} response in the WT was comparable to the preparations from *A. brassicae* (Suppl. Fig. 6). The refractory nature of the three exudate preparations from *A. brassicae* demonstrates that they require the mutated gene for $[Ca^{2+}]_{cyt}$ elevation to CWE/-EPM/EPS (*Ab-CYCAM1*) (Fig. 4A-D). Thus, these preparations contain either the same bioactive compounds, or their signaling events converge early in the plant. The mutant is highly susceptible to *A. brassicae* infection and sensitive to its toxin preparation (Fig. 6A-F). Low concentrations of toxin preparation also induced $[Ca^{2+}]_{cyt}$ elevation, and this response was clearly different from the others. It was detectable in both roots and leaves (Figure 1D). After a stimulus with the above mentioned exudate preparations, a second stimulus with the toxin preparation did not show a refractory nature (Fig. 4A-D). Furthermore, the toxin preparation also induced $[Ca^{2+}]_{cyt}$ elevation in *ab-cycam1* (Fig. 4B-D). Toxins from pathogenic fungi including *A. brassicae* are known to disrupt membranes (Walton, 1996), therefore it is conceivable that this might be the reason for the Ca^{2+} influx into the cytoplasm. If so, this might explain the slow recovery of the signal after having reached the peak (Fig. 1D). The toxin preparation produced the typical disease symptoms of *A. brassicae* on leaves (Fig. 6E-F), while the other Ca^{2+}-inducing exudate preparations did not (data not shown). The *ab-cycam1* is defective in an early defense response against *A. brassicae* and probably to other pathogens. This defense response is relatively mild, when compared to the response to the toxin. The Ca^{2+} response might establish a first line of defense response, which is followed by a second stronger response induced by the toxin or the fungus itself.

Ab-CYCAM1 also plays a role in abiotic (water and drought) stress as demonstrated by the increased sensitivity of *ab-cycam1* seedlings to ABA and mannitol applications (Fig. 8). $[Ca^{2+}]_{cyt}$ elevation is well documented in response to drought stress (Knight et al., 1997; Klüsener et al., 2002). Both ABA- and H_2O_2-induced $[Ca^{2+}]_{cyt}$ elevations in guard cells regulate stomata aperture (Schroeder et al., 2001; Klüsener et al., 2002). Sustained $[Ca^{2+}]_{cyt}$ elevations induced by mannitol is positively correlated to the better tolerance to drought and osmotic stress in Arabidopsis (Knight et al., 1997). Therefore, *Ab-CYCAM1* is involved in biotic and abiotic stress responses.

ABA, SA and JA levels in *ab-cycam1*

To initially characterize the role of *Ab-CYCAM1*, we measured the ABA, SA and JA levels in untreated seedlings and those exposed to *A. brassicae* infections or the toxin. The three

hormones play key roles in mediating disease response to necrotrophic fungal pathogens (Mauch-Mani and Mauch 2005; Bari and Jones, 2009). *ab-cycam1* accumulates higher ABA, SA and JA levels upon *A. brassicae* infection compared to WT (Fig. 9A-C). Therefore, Ab-CYCAM1 interferes with or is involved in these three phytohormone functions. Interaction studies with bitrophic, hemibiotrophic and necrotrophic pathogens on ABA-deficient mutants demon-strated that ABA is a negative regulator of plant defense (Mauch-Mani and Mauch 2005; Fan et al., 2009; Sanchez-Vallet et al., 2012). The hypersusceptibility of *ab-cycam1* to *A. brassicae* and its toxin (Fig. 6A-F) confirms an involvement of Ab-CYCAM1 in the *A. brassicae*-Arabidopsis interaction. The ABA levels were already higher in the two allelic mutants even when not exposed to stress (Fig. 9A), and they were more sensitive to the exogenous ABA application compared to WT (Fig. 8). The major ABA biosynthesis genes *AtBG1*, *NCED3* and *TOC1* were higher induced in *A. brassicae*-exposed mutants than in the WT, whereas *ABA1* and *ABA2* mRNA levels did not show a significant difference (Suppl. Fig. 7). AtBG1, a β-glucosidase located in the endoplasmic reticulum (ER), hydrolyses glucose conjugated biologically inactive ABA to produce active ABA (Lee et al., 2006). NCED3, a nine-cis-epoxycarotenoid dioxygenase (NCED) and timing of CAB expression 1 (TOC1) are involved in *de novo* ABA synthesis (Fan et al., 2009; Ton et al., 2009). Therefore, elevated ABA levels in *A. brassicae*-exposed mutants may be caused by a higher *de novo* synthesis and the conversion of inactive ABA to its active form, and these effects are stimulated in *ab-cycam1* (Fig. 9A and Suppl. Fig. 7).

A. *brassicae* infection also induced SA and SA-responsive genes such as *PRI* and *NPR1* in *ab-cycam1* and WT seedlings (Fig. 9B and Suppl. Fig. 7), and these effects were stronger in the mutant, which again demonstrates the involvement of Ab-CYCAM1 in SA response. SA has both negative and positive role in plant defense against different fungal and bacterial pathogens (c.f. Vlot et al., 2009; Vincente and Plasencia, 2011). The phospholipase Dβ1 *(PLDβ1)* mutant and mutants impaired in phosphatidic acid (PA) biosynthesis were more susceptible to *B. cinerea* infection compared to WT and this was positively correlated to a higher SA level in the infected mutant plants (Zhao et al., 2013), similar to our observations with *ab-cycam1*. PLDβ1 *binds* Ca^{2+}, hydrolyzes phospholipids to generate phosphatidic acid (PA) and is involved in hormone signaling (Zhang et al*.,* 2009) and the response to disease resistance (Zhao et al., 2013). Therefore, elevated Ca^{2+} levels mediated by Ab-CYCAM1 in response to *A. brassicae* stimuli may effect *PLDβ1* expression and PLDβ1 activation.

JA, methyl JA (MeJA) and other bioactive derivatives are important molecules in regulating induced defense responses against necrotrophic pathogen infection (Bari and Jones, 2009; Pieterse et al., 2012). In the present study, *A. brassicae* infection induced constitutively high JA in both WT and *ab-cycam1*, and the induction was higher in the mutant lines (Fig. 9C). Therefore, JA may act as a positive regulator of enhanced susceptibility to *A. brassicae* in *ab-cycam1*. But the biologically active (+)-7-*iso*-JA-Ile level was significantly high in WT compared to mutants (Fig. 9D). The role of JA in disease susceptibility to *Alternaria alternata* f. sp. *lycopersici* (AAL) and its AAL-toxin is well established for tomato (Egusa et al., 2009). Furthermore, JA promoted AAL-toxin-induced cell death through JA insensitive1 (*jai1*) receptor-dependent JA signaling (Zhang et al., 2011a). The JA-responsive genes *MYC2*, *VSP2*, *JAZ1*, *Thi2.1* and *PDF1.2* were upregulated in *A. brassicae* infected WT and *ab-cycam1* and again this stimulation was higher in mutants (Suppl. Fig. 7). Upregulation of a marker gene of the MYC branch (*VSP2*) and of the ERF branch (*PDF1.2*) in *ab-cycam1* suggests that both branches of the JA pathway are involved in the higher susceptibility to *A. brassicae* infections. The *jasmonate regulated gene 21* (JRG21) a common ROS marker gene involved in biotic and abiotic stress and in JA signaling (Mehterov et al., 2012) was also stronger upregulated in *ab-cycam1* than in WT (Suppl. Fig. 7). All these examples indicate that the JA-dependent pathway has a promotional effect on susceptibility of plants to necrotrophic pathogens and that Ab-CYCAM1 is important for JA synthesis and/or signaling in response to *A. brassicae* infection in Arabidopsis.

***ab-cycam1* contains reduced aliphatic glucosinolate levels**

While *A. brassicae* infection stimulated the accumulation of iGLS in both WT and *ab-cycam1,* the accumulation of camalexin was higher in *ab-cycam1* seedlings (Fig. 10A and Suppl. Fig. 5). Higher accumulation of iGLS and camalexin were correlated to the higher disease incidence or susceptibility in different pathosystems (Brader et al., 2006; Bednarek et al., 2009; Su'udi et al., 2011; Wang et al., 2013). Also *PAD3* required for camalexin synthesis was stronger upregulated in the *ab-cycam1* mutants than in WT (Suppl. Fig. 5). The *loh2* mutant perturbed in sphingolipid metabolism was more sensitive to Alternaria toxin and showed a higher expression of the transcription factors, e.g. *ANAC042* and *MYB51* involved in camalexin and iGLS biosynthesis (Mehterov et al., 2012). The stronger regulation of these genes in *ab-cycam1* suggests also an involvement of Ab-CYCAM1 in their regulation. However, aGLS were induced in response to *A. brassicae* infection only in WT and not in *ab-*

cycam1 mutants (Fig. 10B). Furthermore, the aGLS biosynthesis genes *BCAT4* (branched-chain aminoacid aminotransferase 4) and *MYB28* and *MYB29* (Sonderby et al., 2010) were upregulated in WT but not in *ab-cycam1* seedlings (Fig. 10C). These results confirm that aGLS play an important role in defense against *A. brassicae* infection in Arabidopsis and is mediated by Ab-CYCAM1.

Intra- and extra-cellular pools of Ca^{2+} contribute to CWE-, EPM-, EPS- and toxin-induced $[Ca^{2+}]_{cyt}$ elevation

$[Ca^{2+}]_{cyt}$ elevation is resulted from the influx of extracellular apoplatic Ca^{2+} across the plasma membrane and/or the efflux Ca^{2+} from intracellular Ca^{2+} stores in different organelles (Hetherington and Brownlee, 2004). Though pharmacological approaches bear the risk of unspecific off-target effects, it offers the advantage to overcome potential redundancy in target function and allow transient inhibition with minimal pleiotropic effects (Kwaaitaal et al., 2011). In our system, 5 µM staurosporine completely inhibited CWE/EPM/EPS- and toxin- induced $[Ca^{2+}]_{cyt}$ elevation (Fig. 5A-B) indicating that AbCYCAM1 has a kinase domain. Independent studies have also shown the total inhibition of $[Ca^{2+}]_{cyt}$ elevation in cryptogein-tobacco- (Lecourieux et al., 2002) and Myc factor-soybean-suspension cultures (Navazio et al., 2007), Pi-CWE-Arabidopsis root (Vadassery et al., 2009) and ergosterol-tobacco systems (Vatsa et al., 2011). Chelation of extracellular Ca^{2+} by membrane impermeable BAPTA and intracellular Ca^{2+} by membrane permeable neomycin significantly inhibited the $[Ca^{2+}]_{cyt}$ elevation by different *A. brassicae* stimuli (Fig. 5A). Therefore, both extracellular and intracellular Ca^{2+} pools are required for CWE/EPM/EPS/toxin-induced $[Ca^{2+}]_{cyto}$ elevation. Interestingly stauropsorine, BAPTA and neomycin inhibited the seedling growth, especially of mutants (Suppl. Fig. 3). With pharmacological studies, it has been reported that both extra- and intra-cellular Ca^{2+} contribute to $[Ca^{2+}]_{cyt}$ elevation in different systems, e.g. β-glucan-soybean (Mithöfer et al., 1999), Pep-13-parsely (Blume et al., 2000), cryptogein-tobacco (Lecourieux et al., 2002, 2006), Pep-25-Arabidopsis (Hu et al., 2009) and ergosterol-tobacco (Vatsa et al., 2011). Neomycin inhibits IP_3 mediated intracellular Ca^{2+} release in Arabidopsis and Zhang et al. (2011b) could demonstrate the complementary interaction of IP_3 and $[Ca^{2+}]_{cyto}$ in an inositol polyphosphate 1-phosphatase mutant *supo1* in which, elevated IP_3 also elevated $[Ca^{2+}]_{cyt}$ levels and vice versa.

In conclusion, Ab-CYCAM1 is an essential protein involved in $[Ca^{2+}]_{cyt}$-mediated abiotic and biotic stress resistance. *Ab-CYCAM1* mutant is compromised in the induction of aGLS

and the biologically active (+)-7-*iso*-JA-Ile, and accumulated more ABA, which inturn makes *ab-cycam1* more sensitive to *A. brassicae* infection and its toxin. Three exudate preparations from *A. brassicae* activate $[Ca^{2+}]_{cyt}$ in Arabidopsis roots without being toxic by themselves. The overall defense activation of these fractions is low compared to that induced by the fungus itself or by its toxin. We propose a two-step defense response of Arabidopsis roots to *A. brassicae* stimuli, the first one requires Ab-CYCAM1 and $[Ca^{2+}]_{cyt}$ elevation to induce a moderate defense response, while the second step is Ab-CYCAM1 independent, and induces a strong defense response through the fungus or its toxin.

METHODS

Plant material and growth

Transgenic *A. thaliana* expressing cytosolic apoaequorin (Aeq^{cyt}) in Col-0 back-ground (pMAQ2) was a kind gift from Prof. M. Knight (Knight et al., 1991). Mutagenesis was performed using 0.4 and 0.2% EMS (Arabidopsis: A Laboratory Manual, ISBN: 0–87969–573–0). Individual M_2 seeds were grown on HL medium containing 1% agar in square plates (120 x 120 x 16 mm). After stratification at 4°C for 48 h, plates were kept vertically to grow the roots on the surface of the medium and incubated for 16-18 days at 20 ± 1°C under LD conditions and 80 µmol m^{-2} sec^{-1} (Vadassery et al., 2009; Johnson et al., 2011a).

Mutant screening by $[Ca^{2+}]_{cyt}$ measurement

Aequorin based luminescence measurements were performed using 18-day old individual M_2 plants grown on HL medium (Vadassery et al., 2009; Johnson et al., 2011a). pMAQ2 plants (WT) served as control. For $[Ca^{2+}]_{cyt}$ measurements, approximately 70% of the roots per seedling were dissected and incubated overnight in 150 µl of 7.5 µM coelentrazine (P.J.K. GmbH, Germany) in the dark at 20 °C in a 96 well white plate. Bioluminescence counts from roots were recorded as relative light units (RLU) with a microplate luminometer (Luminoskan Ascent, Thermo Electro Corporation, Finland). The mutant screen was performed with the CWE from *A. brassicae*, the putative M_2 mutants were rescued and transferred to pots containing garden soil and vermiculite at 9:1 (v/v) for further screening and validation. The mutant seedlings were grown in a temperature-controlled growth chamber at 20°C under SD condition (8 h light and 16 h darkness) for 4 weeks followed by LD condition (16 h light and 8 h darkness) with a light intensity of 100 µmol m^{-2} sec^{-1} in Aracon tubes. The seeds were

harvested from individual M_3 plants and again screened to obtain homozygote mutants.

Growth conditions and maintenance of *Alternaria brassicae*

The pure culture of *A. brassicae* (FSU-3951) was obtained from Jena Microbial Resource Centre, FSU Jena, Germany and grown on Potato Dextrose Agar (PDA) medium (pH 6.5) at 20±1°C in a temperature controlled chamber under 12/12 h light/dark and 75% relative humidity for 2 weeks. To maintain the virulence, the fungus was inoculated to Arabidopsis seedlings and re-isolated from the infected tissues periodically (Johnson et al., 2013).

Preparation of *A. brassicae* spore suspension

A. brassicae sporulates heavily in Potato Dextrose Broth (PDB; pH 6.5-6.7). A two-week old fungal plug (5 mm diameter) was inoculated to PDB and incubated for 2 weeks. The medium was removed by filtering through 4 layers of sterilized nylon membrane, and the hyphae and spores were washed 3 times with sterile H_2O to remove the residual medium. The spores and hyphae were gently homogenized with 50 ml of sterile H_2O and filtered through four layers of sterilized nylon membrane. The spore concentration was adjusted to 10^4-10^5 colony forming units (cfu) ml^{-1} by serial dilutions or a Haemocytometer. For uniform dispersion of spores, 1-2 drops of Tween-20 was added to 100 ml of spore suspension (Johnson et al., 2013).

Inoculation of *A. brassicae* to roots, seedlings and mature leaves

For root infection, 12-day old seedlings were transferred to fresh PNM plates with a sterilized nylon membrane (for details see Johnson et al., 2011b). A five mm fungal plug from 2-week old *A. brassicae* was kept 1 cm away from the roots. The plates were sealed with parafilm and incubated in a temperature controlled growth chamber under LD condition. Leaf infections were performed 48 h after the transfer of 12-d old seedlings to PNM plates (for details see Johnson et al. 2013). Six leaves in the middle whorl of the seedlings were inoculated with 5 µl of spore suspension containing 10^4-10^5 cfu ml^{-1}. Leaf infection was also performed with detached leaves to assess the susceptibility or resistance of seedlings against *A. brassicae*. Mature leaves were detached from 4-week old plants grown under SD condition at 20°C and 80 µmol m^{-2} sec^{-1}. Sterile Whatmann filter paper was placed on a Petri dish and sterile H_2O was added to just soak the filter paper. Detached leaves were kept on the soaked filter paper and inoculated with 10 µl of the spore suspension containing 10^4 to 10^5 cfu ml^{-1} directly on to leaves. Mock treatment was performed with sterile H_2O. The plates were sealed with parafilm

and incubated under LD conditions as described above. The progression of disease development was determined as PDI at 3, 5, 7 and 10 dai using standard disease intensity grades. For the toxin treatment, 10 µl of the *A. brassicae* toxin prepatation was applied directly on the detached leaves, mock treatment was performed with sterile H_2O.

Preparation of the CWE from *A. brassicae*

The CWE was prepared according to the protocol of Johnson et al. (2011a). Mycelia from 15-day old PDB cultures were harvested and used for the CWE preparation. The CWE was further purified by passing it through a reversed phase Supelclean LC-18 SPE cartridge (bed wt., 10 g; volume, 60 ml; pore size, $60A^0$; cat. no. 57136 of Sigma-Aldrich, Taufkirchen, Germany). The fractions were identified by $[Ca^{2+}]_{cyt}$ measurements, combined and used for growth assays (Johnson et al., 2011a).

Preparation of exudates preparations from mycelia (EPM) and germinating spores (EPS) of *A. brassicae*

A. brassicae mycelium, propagated on PDB for 14 days, was filtered through four layers of sterilised nylon membrane and intensively washed seven times with sterile H_2O to remove the medium and spores. After air drying of the mycelium for 1 h, it was resuspended in 100ml sterile H_2O and incubated at 20^0C in a horizontally rotating shaker at 60 rpm. After 48 h, the mycelium was removed from the water by filtering through 4 layers of sterile nylon membrane, then through 2 layers of filter paper and finally filter-sterilized using a 0.22 µ filters to get EPM. For preparing EPS, the spore suspension was prepared with an inoculum concentration of 10^7-10^8 cfu ml^{-1} (Johnson et al., 2013) and incubated for 48 h at 20^0C in a horizontally rotating shaker at 60 rpm. By this time, more than 90% of spores are germinated and filtered through 4 layers of sterile nylon membrane, then through 2 layers of filter paper and finally filter-sterilized using a 0.22 µ filters. EPM and EPS were further purified using a reverse phased Supelclean LC-18 cartridge to obtain the active and partially pure fractions (Johnson et al., 2011a), which were used for $[Ca^{2+}]_{cyt}$ measurement and growth assays.

***A. brassicae* toxin preparation**

A toxin preparation from *A. brassicae* culture filtrate was extracted as described by Vidhyasekaran et al. (1997). The crude toxin preparation was further purified by passing it through a Sephadex G100 column and the active fractions were collected, concentrated and

lyophilised. The powder was resuspended in sterile H_2O and further purified by passing it through a reversed phase Supelclean LC-18 SPE cartridge. The active fractions were collected and used as stimulus for $[Ca^{2+}]_{cyt}$ measurement and growth assays. Further fractionation of CWE, EPM, EPS and the toxin is described along with Suppl. Fig. 2.

Pharmacological analyses

All chemicals used for pharmacological analysis were obtained from Sigma, Germany except staurosporine which was purchased from LC Laboratories MA 01801, USA. Stock solutions were prepared either in sterile H_2O or in DMSO. The influence of the DMSO in the final concentration after chemical dilution was tested as an additional control.

Germination, growth of seedling and root assays

The surface-sterilized seeds of wild type (pMAQ2) and the *ab-cycam1* mutant were placed on MS medium. For drought and water stress experiments, different concentrations of mannitol were added before autoclaving and ABA were added after autoclaving. WT and *ab-cycam1* mutants were also grown on MS medium alone or supplemented with the $[Ca^{2+}]_{cyt}$ -inducing fractions or different chemicals at concentrations mentioned in the text. After cold treatment at 4°C for 48 h, plates were incubated at 20°C under LD condition, 80 µmol m^{-2} sec^{-1} for 10-20 days depending on the experiments.

Measurement of photosynthesis parameter

Two-week old *Arabidopsis* seedlings grown on MS medium were dark-adapted for 20 min and the chlorophyll fluorescence was measured at room temperature using a video imaging with a pulse amplitude-modulated FluorCam 700F (Photon System Instruments, Brno, Czech Republic). Program parameters of FluorCam were essentially according to Wagner et al. (2008). Photosynthesis parameters; quantum yield of PSII (Φ_{PSII}), maximum quantum yield of PSII (Fv/Fm), photochemical quenching (qP) and non-photochemical quenching (NPQ) was calculated based on Maxwell and Johnson (2000). False color images of the seedlings in plates were obtained as described by Wagner et al. (2008). Chl fluorescence images representing Fs/Fm values are shown, whereas blue represent low F_s/F_m values above a threshold of 0.06 and red represents high F_s/F_m values with an upper threshold limit of 0.17.

Phytohormone, camalexin and glucosinolates measurements by HPLC

For phytohormones measurement, approximately 100 mg of leaf material was extracted with

1.2 ml of methanol containing internal standards of different phytohormones. For camalexin and glucosinolates measurements, 40-50 mg of leaf material was extracted with 80% methanol. Phytohormones, camalexin and glucosinolates are measured using Agilent 1100 HPLC system, Agilent Technologies, Waldbronn, Germany. The detailed protocol is described in the supplemental material 1.

Semi quantitative reverse transcription-PCR Analysis

Total RNA was extracted using RNeasy Plant Mini kit with DNAse I treatment (Qiagen). cDNA was synthesized using the Omniscript cDNA synthesis kit (Qiagen) with 1 µg RNA. Semi-quantitative RT-PCR was performed using 1:4 fold diluted cDNA. The oligonucleotide primers are given in Supplemental Material 2. The mRNA levels for each cDNA probe were normalized with respect to the *GAPDHC* message levels and expressed relative to the WT control.

ACKNOWLEDGEMENTS

J.M.J. was supported by the IMPRS and FSU Jena. We thank Prof. Scheel for providing flg22, Sarah Mußbach and Claudia Röppischer for their excellent technical assistance.

REFERENCES

Bains, P.S. and Tewari, J.P. (1987) Purification, chemical characterization and host-specificity of the toxin produced by *Alternaria brassicae*. *Physiol Mol Plant Pathol*, **30**, 259-271.

Bari, R. and Jones, J.D.G. (2009) Role of plant hormones in plant defence responses. *Plant Mol Biol*, **69**, 473-488.

Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina. A and Schulze-Lefert, P. (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science*, **323**, 101-106.

Blume, B., Nürnberger, T., Nass, N. and Scheel, D. (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defence in parsley. *Plant Cell*, **12**, 1425-1440.

Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol*, **60**, 379-406.

Bourque, S., Binet, M.N., Ponchet, M., Pugin, A. and Lebrun-Garcia, A. (1999) Characterization of the cryptogein binding sites on plant plasma membranes. *J Biol Chem*, **274**, 34699-34705.

Brader, G., Mikkelsen, M.D., Halkier, B.A. and Tapio Palva, E. (2006) Altering glucosinolate profiles modulates disease resistance in plants. *Plant J*, **46**, 758-767.

Clapham, D.E. (2007) Calcium signaling. *Cell*, **131**, 1047-1058.

Dodd, A.N., Kudla, J. and Sanders, D. (2010) The language of calcium signaling. *Annu Rev Plant* Biol, **61**, 593-620.

Egusa, M., Ozawa, R., Takabayashi, J., Otani, H. and Kodama, M. (2009) The jasmonate signaling pathway in tomato regulates susceptibility to a toxin-dependent necrotrophic pathogen. *Planta*, **229**, 965-976.

Fan, J., Hill, L., Crooks, C., Doerner, P. and Lamb, C. (2009) Abscisic acid has a key role in modulating diverse plant-pathogen interactions. *Plant Physiol*, **150**, 1750-1761.

Frei dit Frey, N., Mbengue, M., Kwaaitaal, M., Nitsch, L., Altenbach, D., Häweker, H., Lozano-Duran, R., Njo, M.F., Beeckman, T., Huettel, B., Borst, J.W., Panstruga, R. and Robatzek, S. (2012) Plasma membrane calcium ATPases are important components of receptor-mediated signaling in plant immune responses and development. *Plant Physiol*, **159**, 798-809.

Halkier, B.A. and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol*, **5**, 303-333.

Hetherington, A.M. and Brownlee, C. (2004) The generation of Ca^{2+} signals in plants. *Annu Rev Plant Biol*, **55**, 401-427.

Hu, X., Neill, S.J., Yang, Y. and Cai, W. (2009) Fungal elicitor Pep-25 increases cytosolic calcium ions, H_2O_2 production and activates the octadecanoid pathway in *Arabidopsis thaliana*. *Planta*, **229**, 1201-1208.

Jeworutzki, E., Roelfsema, M.R.G., Anschütz, U., Krol, E., Elzenga J.T.M., Felix, G., Boller, T., Hedrich, R. and Becker, D. (2010) Early signaling through the Arabidopsis pattern recognition receptors FLS2 and EFR involves Ca^{2+}-associated opening of plasma membrane anion channels. *Plant J*, **62**, 367-378.

Johnson, J.M., Nongbri, P.L., Sherameti, I. and Oelmüller, R. (2011a) Calcium signaling and cytosolic calcium measurements in plants. *Endocyt Cell Res*, **21**, 64-76.

Johnson, J.M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Varma, A. and Oelmüller, R. (2011b) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation - A model system to study plant beneficial traits. *Endocyt Cell Res*, **21**, 101-113.

Johnson, J.M., Sherameti, I., Nongbri P.L., and Oelmüller, R. (2013) Standardized conditions to study beneficial and nonbeneficial traits in the *Piriformospora indica*/*Arabidopsis thaliana* interaction. In. *Piriformospora indica - Sebacinales and their biotechnological applications, Soil Biology*, **33**, 325-343. A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg Germany.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323-329.

Klüsener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V. and Schroeder, J.I. (2002) Convergence of calcium signalling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. *Plant Physiol*, **130**, 2152-2163.

Knight, H., Trewavas, A.J. and Knight, M.R. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J*, **12**, 1067-1078.

Knight, M.R., Campbell, A.K., Smith, S.M. and Trewavas, A.J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature*, **352**, 524-526.

Kudla, J., Batistic, O. and Hashimoto, K. (2010) Calcium signals: the lead currency of plant information processing. *Plant Cell*, **22**, 541-563.

Kwaaitaal, M., Huisman, R., Maintz, J., Reinstädler, A. and Panstruga, R. (2011) Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP induced calcium influx in *Arabidopsis thaliana*. *Biochem J*, **440**, 355-365.

Larkindale, J. and Knight, M.R. (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiol*, **128**, 682-695.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R. and Pugin, A. (2002) Analysis and effects of cytosolic free calcium elevation in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell*, **14**, 2627-2641.

Lecourieux, D., Ranjeva, R. and Pugin, A. (2006) Calcium in plant defence-signaling pathways. *New Phytol*, **171**, 249-269.

Lee, K.H., Piao, H.L., Kim, H.Y., Choi, S.M., Jiang, F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J., and Hwang, I. (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell*, **126**, 1109-1120.

Maxwell, K. and Johnson, G.N. (2000) Chlorophyll fluorescence - a practical guide. *J Exp Bot*, **51**: 659-668.

Mauch-Mani, B. and Mauch, F. (2005) The role of abscisic acid in plant-pathogen interactions. *Curr Opin Plant Biol*, **8**, 409-414.

Mehterov, N., Balazadeh, S., Hille, J., Toneva, V., Müller-Röber, B. and Gechev, T. (2012) Oxidative stress provokes distinct transcriptional responses in the stress-tolerant *atr7* and stress-sensitive *loh2 Arabidopsis thaliana* mutants as revealed by multi-parallel quantitative real-time PCR analysis of ROS marker and antioxidant genes. *Plant Physiol Biochem*, **59**: 20-29.

Meindl, T., Boller, T. and Felix, G. (2000) The bacterial elicitor flagellin activates its receptor in tomato cells according to the address-message concept. *Plant Cell*, **12**, 1783-1794.

Millet, Y.A., Danna, C.H., Clay, N.K., Songnuan, W., Simon, M.D., Werck-Reichhart, D. and Ausubel, F.M. (2010) Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. *Plant Cell*, **22**, 973-990.

Mithöfer, A., Ebel, J., Bhagwat, A.A., Boller, T. and Neuhaus-Url, G. (1999) Transgenic aequorin monitors cytosolic transients in soybean cells challenged with β-glucan or chitin elicitors. *Planta*, **207**, 566-574.

Mithöfer, A. and Mazars, C. (2002) Aequorin-based measurements of intracellular Ca^{2+}-signatures in plant cells. *Biol Proced Online*, **4**, 105-118.

Moebius, N. and Hertweck, C. (2009) Fungal phytotoxins as mediators of virulence. *Curr Opin Plant Biol*, **12**, 390-398.

Müller, J., Staehelin, C., Xie, Z-P., Neuhaus-Url, G. and Boller T. (2000) Nod factors and chitooligomers elicit an increase in cytosolic calcium in aequorin-expressing soybean cells. *Plant Physiol*, **124**, 733-740.

Navazio, L., Moscatiello, R., Genre, A., Novero, M., Baldan, B., Bonfante, P. and Mariani, P. (2007) A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. *Plant Physiol*, **144**, 673-681.

Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W., Hahlbrock, K. and Scheel D. (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. *Cell*, **78**, 449-460.

Pedras, M.S.C. and Khallaf, I. (2012) Molecular interactions of the phytotoxins destruxin B and sirodesmin PL with crucifers and cereals: Metabolism and elicitation of plant defenses. *Phytochem*, **77**, 129-139.

Pieterse, C.M.J., van der Does, D., Zamioudis, C., Leon-Reyes, A. and van Wees, S.C.M. (2012) Hormonal modulation of plant immunity. *Annu Rev Cell Dev Biol*, **28**, 489-521.

Ranf, S., Grimmera, J., Pöschl, Y., Pecher, P., Chinchilla, D., Scheel, D. and Lee, J. (2012) Defense-related calcium signaling mutants uncovered *via* a quantitative high-throughput screen in *Arabidopsis thaliana*. *Mol Plant*, **5**, 115-130.

Reddy, A.S., Ali, G.S., Celesnik, H. and Day, I.S. (2011) Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *Plant Cell*, **23**, 2010-2032.

Reddy, V.S. and Reddy, A.S.N. (2004) Proteomics of calcium-signaling components in plants. *Phytochem*, **65**, 1745-1776.

Sánchez-Vallet, A., López, G., Ramos, B., Delgado-Cerezo, M., Riviere, M.-P., Liorente, F., Fernández, P.V., Miedes, E., Estevez, J.M., Grant, M. and Molina, A. (2012) Disruption of abscisic acid signalling constitutively activates Arabidopsis resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. *Plant Physiol*, **160**, 2109-2124.

Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell*, **14**, S401-S417.

Schroeder, J.I., Kwak, J.M. and Allen, G.J. (2001) Guard cell abscisic acid signalling and engineering drought hardiness in plants. *Nature*, **410**, 327-330.

Sonderby, I.E., Burow, M., Rowe, H.C., Kliebenstein, D.J. and Halkier, B.A. (2010) A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in Arabidopsis. *Plant Physiol*, **153**, 348-363.

Su'udi, M., Kim, M.G., Park, S.R., Hwang, D.J., Bae, S.C. and Ahn, I.P. (2011) Arabidopsis cell death in compatible and incompatible interactions with *Alternaria brassicicola*. *Mol Cells*, **31**, 593-601.

Ton, J., Flors, V. and Mauch-Mani, B. (2009) The multifaceted role of ABA in disease resistance. *Trends Plant Sci*, **14**, 310-317.

Vadassery, J., Ranf, S., Drewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J. and

Oelmüller R. (2009) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *Plant J*, **59**, 193-206.

Vadassery, J., Reichelt, M., Hause, B., Gershenzon, J., Boland, W. and Mithöfer, A. (2012) CML42-mediated calcium signaling coordinates responses to Spodoptera herbivory and abiotic stresses in Arabidopsis. *Plant Physiol*, **159**, 1159-1175.

Vatsa, P., Chiltz, A., Bourque, S., Wendehenne, D., Garcia-Brugger, A. and Pugin, A. (2011) Involvement of putative glutamate receptors in plant defence signaling and NO production. *Biochimie*, **93**, 2095-2101.

Vidhyasekaran, P., Ruby Ponmalar, T., Samiyappan, R., Velazhahan, R.,Vimala, R., Ramanathan, A., Paranidharan, V. and Muthukrishnan, S. (1997) Host-specific toxin production by *Rhizoctonia solani*, the rice sheath blight pathogen. *Phytopathol*, 87, 1258-1263.

Vicente, M.R. and Plasencia, J. (2011) Salicylic acid beyond defence: its role in plant growth and development. *J Exp Bot*, **62**, 3321-3338.

Vlot, A.C., Dempsey, D.A. and Klessig, D.F. (2009) Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol*, **47**, 177-206.

Wagner, R., Dietzel, L., Bräutigam, K., Fischer, W. and Pfannschmidt, T. (2008) The long-term response to fluctuating light quality is an important and distinct light acclimation mechanism that supports survival of *Arabidopsis thaliana* under low light conditions. *Planta*, **228**, 573-587.

Wang, Y., Bouwmeester, K., van de Mortel, J.E., Shan, W. and Govers, F. (2013) A novel Arabidopsis-oomycete pathosystem: differential interactions with *Phytophthora capsici* reveal a role for camalexin, indole glucosinolates and salicylic acid in defence. *Plant Cell Environ*, **36**, 1192-1203.

Walton, J. D. (1996) Host-selective toxins: Agents of compatibility. *Plant Cell*, **8**, 1723-1733.

Zhang, J., Vanneste, S., Brewer, P.B., Michniewicz, M., Grones, P., Kleine-Vehn, J., Löfke, C., Teichmann, T., Bielach, A., Cannoot, B., Hoyerová, K., Chen, X., Xue, H.W., Benková, E., Zažímalová, E. and Friml, J. (2011b) Inositol trisphosphate-induced Ca^{2+} signaling modulates auxin transport and PIN polarity. *Dev Cell*, **20**, 855-866.

Zhang, L.P., Jia, C.G., Liu, L.H., Zhang, Z.M., Li, C.Y., and Wang, Q.M. (2011a) The involvement of jasmonates and ethylene in *Alternaria alternata* f. sp. *lycopersici* toxin-induced tomato cell death. *J Exp Bot*, **62**, 5405-5418.

Zhang, Y., Zhu, H., Zhang, Q., Li, M., Yan, M., Wang. R., Wang, L., Welti, R., Zhang, W. and Wan, X. (2009) Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in Arabidopsis. *Plant Cell*, **21**, 2357-2377.

Zhao, J., Devaiah, S. P., Wang, C., Li, M., Welti, R. and Wang, X. (2013) Arabidopsis phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens. *New Phytol*, **199**, 228-240.

5. Discussion

Molecular communications between plant and the interacting microbes commence immediately after the microbes make contact with the plant surface or in some case even before their physical contact. Beneficial and pathogenic fungi release many compounds e.g. oligosaccharides, peptides/proteins, glycoproteins, lipids and lipoproteins for its interaction with plant cells. Some of these compounds may act as beneficial microbe-associated molecular patterns (bMAMP) as in *P. indica* or pathogen-associated molecular patterns (PAMPs) or effectors as in *A. brassicae*. bMAMPs, PAMPs and effectors are evolutionarily conserved molecules in microbes which help them to lead their microbial life style either as endosymbionts or as pathogens. bMAMPs, PAMPs and effectors also act as molecular cues for successful recognition and subsequent downstream responses leading to either beneficial or pathogenic interactions. The vast structural diversity of fungal factors suggests that there is no common microbial motif to initiate the molecular dialogues with the host cells, and that plants might have evolved versatile perception systems for microbe-derived structures. In this thesis, the effects of a *P. indica*-derived factor on growth promotion, and of *A. brassicae*-derived factors on plant defense are analysed.

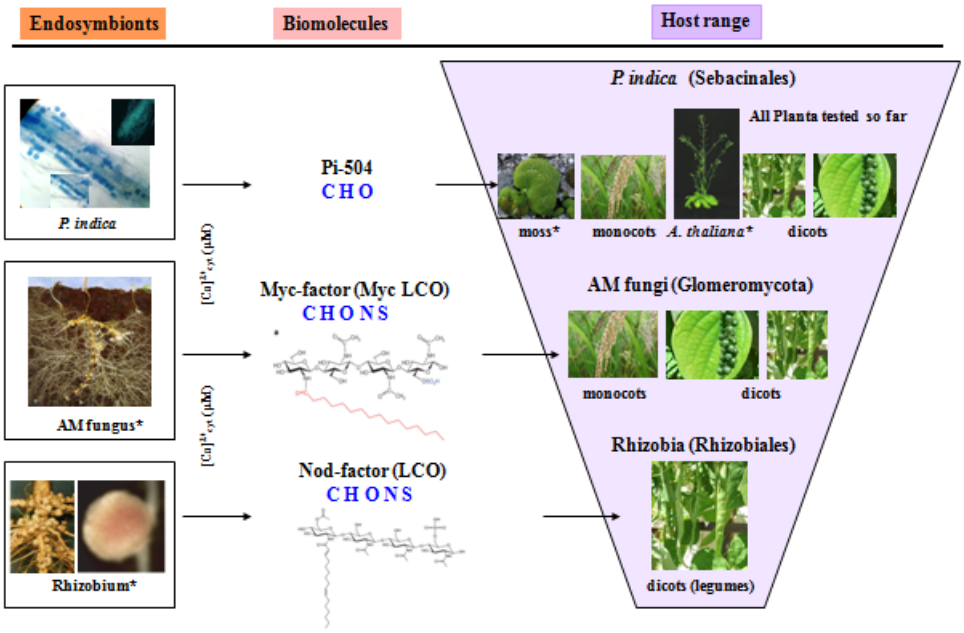
5.1 Pi-504 is the active factor in the cell wall extract of *P. indica* and Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation promotes growth in Arabidopsis

bMAMPs- and PAMPs-dependent $[Ca^{2+}]_{cyt}$ changes play a crucial role in cell signal transduction, modulating an array of biological processes involved in growth, development, symbiosis and defense. *A. thaliana* (Col-0) expressing the bioluminescent Ca^{2+} binding apoaequorin in the cytosol is used to study the role of $[Ca^{2+}]_{cyt}$ changes in the beneficial interaction between *A. thaliana* and *P. indica*. Perception of beneficial microbes and their bMAMPs leading to $[Ca^{2+}]_{cyt}$ elevation is one of the earliest physiological events that can be measured in plant cells (Navasio *et al*. 2007; Kosuta *et al*. 2008; Vadassery *et al*. 2009a). The active component(s) from the fungal CWE induce(s) $[Ca^{2+}]_{cyt}$ elevation in roots and stimulates the growth of Arabidopsis (Vadassery *et al*. 2009a; Johnson *et al*. 2011a, 2013b) and Chinese cabbage seedlings (Lee *et al*. 2011; Johnson *et al*. 2013a), as the fungus does. The biologically active component in the Pi-CWE was purified as a trisaccharide ($C_{18}H_{32}O_{16}$) with *m/z* of 505.1748 (Pi-504). Pi-504 induces $[Ca^{2+}]_{cyt}$ elevation in a dose-dependent manner with a distinct Ca^{2+} signature, i.e. a lag time, peak time, duration and shape in roots. Acid hydrolysis

cleaved the trisaccharide into monomers, glucose and galactose, which did not induce $[Ca^{2+}]_{cyt}$ elevation. Pi-504 also promoted the growth of tobacco seedlings, as the fungus does. Therefore, growth promotion can be uncoupled from root colonisation by the fungus. Growth promotion by Pi-504 was less compared to the fungus-induced growth promotion in Arabidopsis and tobacco seedlings, as the fungus constantly interacts with and releases the signaling molecule to the host plants. Two well characterized bMAMPs, the Myc factor lipochitooligosaccharides from arbuscular mycorrhizal fungi (Maillet *et al*. 2011) and the Nod factor lipochitooligosaccharides from rhizobia (Ehrhardt *et al*. 1996) also induce $[Ca^{2+}]_{cyt}$ elevation in their respective host roots (Navazio *et al*. 2007; Kosuta *et al*. 2008). The endosymbiotic interaction of *P. indica* with plant roots results in acquisition of more nitrogen by modulating N_2 metabolism and phosphate uptake from the environment (Sherameti *et al*. 2005; Yadav *et al*. 2010). Like the fungus, Pi-504 stimulates *Nia1* and *Nia2* expression, genes involved in nitrogen metabolism, and *Pht1.1* and *Pht1.5* expression, genes involved in phosphate uptake and transport. Pi-504 may be a highly conserved bMAMP in *P. indica* which helps the fungus to perform the endosymbiotic lifestyle in a variety of host plants. This should be confirmed genetically with the mutant of *P. indica* which lacks Pi-504. Pi-504 is a simple oligosaccharide and composed of carbon, hydrogen and oxygen, which form the back-borne of many biomolecules. The small and simple nature of Pi-504 may helps the fungus to colonise the such a broad host spectrum including bryophytes, pteridophytes, gymnosperms and a large number of angiosperms with agriculturally and horticulturally important crop plants (c.f. Oelmüller *et al*. 2009; Franken 2012; Qiang *et al*. 2012a; Varma *et al*. 2012), in contrast to the complex Myc- and Nod-factors (Figure 6), which may be responsible for their host specificity. *P. indica* can also survive and form endosymbiosis in diverse and extreme agroclimatic conditions, for example with xerophytes grown in deserts and conifers grown in Himalayas under extreme cold conditions.

5.2 *P. indica*-dervived auxin is not involved in growth promotion

Auxins are crucial for lateral root production, cell expansion, cell division and cell differentiation (Ludwig-Müller and Güther 2005). Microbes interfere with the plant auxin synthesis, metabolism, signaling and transport (Vadassery *et al*. 2008; Lee *et al*. 2011; Johnson *et al*. 2013a). Sirrenberg *et al*. (2007) proposed that auxin released by *P. indica* is responsible for growth promotion and the morphological changes in the roots. However, under our growth condition, both free and conjugated indole-3-acetic acid (IAA) levels were



* pictures from internet

Figure 6. Comparison of different beneficial microbe associated molecular patterns (bMAMP) from three well known endosymbionts which induce cytosolic calcium signaling in their respective host plants. Pi-504 from *P. indica* is a simple and low molecular weight trisaccharide with carbon, hydrogen and oxygen, and fungus has a wide host range; whereas Myc- (mycorrhiza) factor and Nod- (nodulation) factors are lipochito-oligosaccharides (LCO) with more elemental compositon, e.g. carbon, hydrogen, oxygen, nitrogen and sulphur elemental composition with complex structures have limited hosts.

not changed in colonized and mock-treated Arabidopsis seedlings even though the fungus promoted growth. Moreover, large scale microarray analysis and gene expression studies in Arabidopsis roots colonized by *P. indica*, did not show induction of genes involved in the biosynthesis, signaling and transport of auxin (Vadassery *et al*. 2008; Lee *et al*. 2011). In addition, mutants with reduced auxin levels (*ilr1-1*, *nit1-3*, *tfl2*, *cyp79 b2b3, aux1, aux1 axr4, rhd6*) responded to *P. indica*, which indicated that severe alterations in auxin homeostasis in Arabidopsis do not prevent the growth response to *P. indica* (Vadassery *et al*. 2008; Lee *et al*. 2011; Johnson *et al*. 2013a). Interestingly, the auxin overproducer *sur1-1* with a dwarf phenotype responds more sensitively to *P. indica* and its dwarf phenotype is almost rescued by the fungus. *P. indica* reduces the free auxin level by converting it to its inactive conjugates (Vadassery *et al*. 2008). These results strongly suggest that the *P. indica* mediated growth promotion in Arabidopsis is largely independent of auxin biosynthesis and signaling, although all growth processes require auxin. We conclude that at least auxin released by the fungus is not responsible for the growth response of the plants, which is further supported by the observation that exogenously applied auxin cannot replace the fungus. Finally, a HPLC-

purified Pi-CWE and Pi-WDE (water diffusible extract) from the fungus, which do not contain auxin, promoted growth of Arabidopsis and Chinese cabbage seedlings (Lee *et al*. 2011; Johnson *et al*. 2013a). However, auxin plays an important role in *P. indica* mediated growth promotion in Chinese cabbage and barley, as the auxin level increased and auxin-related genes were upregulated after the colonization (Schäfer *et al*. 2009a, b; Sun *et al*. 2010; Lee *et al*. 2011; Hilbert *et al*. 2012). Apparently, auxin signaling is more important for *P. indica*-induced growth promotion in Chinese cabbage than in Arabidopsis (Lee *et al*. 2011). Furthermore, an exudate component from *P. indica* but not auxin stimulated growth of Chinese cabbage and Arabidopsis seedlings (Lee *et al*. 2011; Johnson *et al*. 2013a). We propose that activation of auxin biosynthesis and signaling in the roots but not the auxin produced by the fungus is the cause for the *P. indica*- and Pi-504- mediated growth phenotype in Chinese cabbage.

5.3 Establishment of a qualitative high-throughput Ca^{2+}-based screening system and isolation of $[Ca^{2+}]_{cyt}$ mutants non-responsive to Pi-504

A non-destructive high throughput mutant screening method with 96-well plate in combination with a plate-reader luminometer was established to isolate mutants which do not respond to Pi-504 with regard to $[Ca^{2+}]_{cyt}$ elevation (Nongbri *et al*. 2012; Johnson and Oelmüller 2013; for the detailed protocol in Vadassery *et al* 2009a; Johnson *et al* 2011a). By this method 96 samples could be measured within 1 h. Screening of EMS-mutagenized M_2 populations of pMAQ2 resulted in the isolation of mutants which do not show $[Ca^{2+}]$ elevation to Pi-504 (*pi-cycam* - cytosolic calcium mutant to Pi-504). More than 2.40.000 seedlings were screened to obtain mutants which do not show any pleotropic phenotype. To determine the inheritance pattern of the mutated gene(s), the mutants were backcrossed to their ancestor WT (pMAQ2) which resulted in the complete restoration of $[Ca^{2+}]_{cyt}$ elevation to Pi-504 in ~25% of F_2 progenies. Therefore, the mutation is a recessive and qualitative trait. To determine whether the mutants are allelic, complementation analysis was performed by intercrossing all four mutants. All F_1 progenies did not show $[Ca^{2+}]_{cyt}$ elevation to Pi-504 which demonstrates that the four mutants are allelic. A future task is to identify the mutated gene by map-based cloning strategies. To initiate this, I crossed the mutant with WT La-*er* (L*er*-0) and selected those F_2 plants with La-*er* phenotype which did not show $[Ca^{2+}]_{cyt}$ elevation to Pi-504. Chromosomal marker analysis (Bell and Ecker 1994; Lukowitz *et al*. 2000) suggests that the mutated gene is linked to chromosome 1 (Table 1).

Table 1. Chromosome mapping of *Pi-CYCAM* - *cycam* was crossed with WT (La-*er*) and selected only those F_2 plants with La-*er* phenotype having *Pi-CYCAM* (36 plants) for the chromosome mapping. Both pMAQ2 (Col-0) and La-*er* with aequorin were used as the positive control.

		Markers	PCR product (C for Col-0) or (L for La-*er*)		
			pMAQ2 (Col-0)	(La-*er*)	Mapping Population
Chromosome 1	1	nga 280	C	L	C
	2	nga 111	C	L	C
	3	nga 128	C	L	C
	4	nga 59	C	L	C
	5	nga 63	C	L	C
	6	T27K 12-Sp6	C	L	C
Chromosome 2	1	nga 168	C	L	L
	2	nga 1145	C	L	L
	3	nga 1126	C	L	L
	4	nga 361	C	L	L
	5	Ath BIO2b	C	L	L
Chromosome 3	1	nga 162	C	L	C+L
	2	nga 6	C	L	C+L
	3	nga 112	C	L	C+L
	4	nga 126	C	L	C+L
	5	nga 172	C	L	C+L
	6	Ath GAPAb	C	L	C+L
	7	nga 707	C	L	C+L
Chromosome 4	1	nga 8	C	L	L
	2	nga 12	C	L	L
	3	nga 1111	C	L	L
	4	nga 1139	C	L	L
	5	nga 1107	C	L	L
Chromosome 5	1	nga 76	C	L	C+L
	2	nga 151	C	L	C+L
	3	nga 225	C	L	C+L
	4	nga 249	C	L	C+L
	5	nga 106	C	L	C+L
	6	nga 139	C	L	C+L
	7	Ath PHYC	C	L	C+L

5.4 *Pi-CYCAM* mutants are impaired in growth promotion to Pi-504 and *P. indica*

Large scale screening of an EMS mutagenized seedlings resulted in the isolation of *pi-cycam*

which do not induce $[Ca^{2+}]_{cyt}$ elevation in response to Pi-504. Interestingly, Pi-504 also fails to promote growth in mutants. We propose that the Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation is essential for growth promotion in Arabidopsis seedlings. To our knowledge, this is the first genetic evidence linking the $[Ca^{2+}]_{cyt}$ elevation and growth promotion in endosymbiosis. *Pi-CYCAM* mutants also showed more than 70% reduced growth promotion to *P. indica* compared to WT in our cocultivation experiment (detailed protocol in Johnson *et al*. (2011b)). It is assumed that the fungus may be producing more than one compound that are involved in growth promotion or also using some other mechanisms to stimulate growth promotion. In the present study, the mRNA levels of *Nia1* and *Nia2* involved in nitrogen metabolism (Sherameti *et al*. 2005) and *Pht1.1* and *Pht1.5* involved in phosphate uptake (Yadav *et al*. 2010) were upregulated in WT compared to *pi-cycam*. Therefore, the genes involved in primary metabolism of nitrogen and phosphorus are the direct target of the fungus and Pi-504. The fitness of the seedlings to photosystems by measuring various chlorophyll (chl) fluorescence parameters, for example, maximum quantum yield of photosystem II (PSII) (F_v/F_m), quantum yield of PSII (Φ_{PSII}), photochemical quenching or proportion of open PS II (qP) and non-photochemical quenching (NPQ) (Maxwell and Johnson 2000) shows that Φ_{PSII}, qP and NPQ are significantly decreased in *pi-cycam* compared to WT. The efficiency of the photosynthetic electron transport (Φ_{PSII}, qP) and the ability of heat dissipation of photochemical energy (NPQ) are impaired to a lesser extent in the chloroplasts of *pi-cycam*. Therefore, we propose that the Pi-504-induced $[Ca^{2+}]_{cyt}$ elevation is directly involved in enhanced photosynthesis, nitrogen metabolism and phosphate uptake which, in turn leads to growth promotion in *A. thaliana* seedlings (Figure 7).

5.5 Beneficial and pathogenic fungal components require CYCAM for $[Ca^{2+}]_{cyt}$ elevation

The EMS-mutagenized M_2 populations of pMAQ2 were also screened with a CWE from *A. brassicae*. This necrotrophic fungus is highly specific to crucifers and causes severe crop loss (Bains and Tewari 1987). We isolated homozygous mutants which do not respond to the CWE. Interestingly, the homozygous mutants (*ab-cycam* - cytosolic calcium mutants to Ab-CWE), which do not respond to the CWE from *A. brassicae* responded normally to the toxin preparation from *A. brassicae*, but did not respond to the exudates preparation from mycelium (EPM) and germinating spores (EPS) from *A. brassicae* and to Pi-504 from *P. indica*. Later, we found that both *pi-cycam* and *ab-cycam* are allelic and the mutants are renamed as *cycam* (cytosolic calcium mutant). *cycam* was further tested with CWE and EPM preparations from

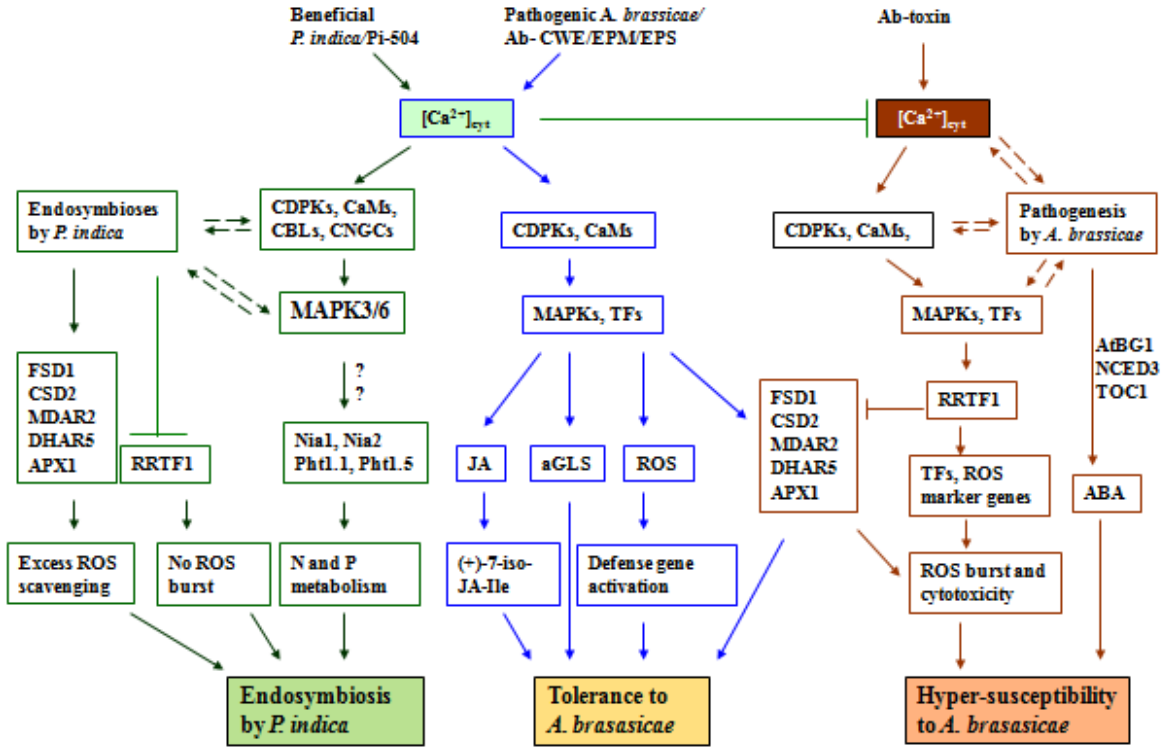


Figure 7. Proposed model showing the role of cytosolic Ca^{2+} signaling in beneficial and pathogenic fungal interactions in *Arabidopsis thaliana*. Cytosolic Ca^{2+} signaling cascades by *P. indica* and Pi-504 leading to endosymbiosis are shown in green lines and boxes. A. *brassicae*-CWE/EPM/EPS-induced cytosolic Ca^{2+} signaling resulting in tolerance to *A. brassicae* infection is shown in blue lines and boxes. Pi-504- and Ab-CWE/EPM/EPS- induced cytosolic Ca^{2+} signaling cascades leading to growth promotion and plant defense respectively, repress the Ab-toxin-induced Ca^{2+} signaling cascades which leads to hypersusceptibility to *A. brassicae* infection mainly by deregulating the tight control of ROS production. Ab-toxin-induced Ca^{2+} signaling cascades leading to hypersusceptibility to *A. brassicae* infection are shown in brown lines and boxes (Ab, *Alternaria brassicae*; ABA, abscisic acid; aGLS, aliphatic glucosinolates; APX, Ascorbate peroxidase; At-BG1, *Arabidopsis thaliana* β-glucosidase; $[Ca^{2+}]_{cyt}$, cytosolic calcium; CaM, calcium calmodulin; CBL, calcineurin B-like calcium sensor; CDPK, calcium dependent protein kinase; CNGC, cyclic nucleotide gated ion channel; CSD, copper superoxide dismutase; CWE, cell wall extract; DHAR, dehydroascorbate reductase; EPM, exudates preparation from mycelium; EPS, exudates preparation from germinating spores; FSD, iron superoxide dismutase; JA, jasmonic acid; ((+)-7-*iso*-JA-Ile), (+)-7-*iso*-jasmonoyl-1-isoleucine; MAPK, mitogen activated protein kinase; MDAR, monodehydroascorbate reductase; NCED, nine-cis-epoxycarotenoid dioxygenase; Nia, nitrate reductase; Pht, phosphate transporter; Pi, *Piriformospora indica*; ROS, reactive oxygen species; RRTF1, redox responsive transcription factor1; TF, transcription factor; TOC, timing of CAB expression).

another endophytic beneficial fungus, *Acremonium alternatum* (Jäschke *et al*. 2010), and different pathogenic fungi, e.g. *Rhizoctonia solani* (FSU 1137), *Fusarium solani* (FSU 5059) and the oomycete, *Phytophthora parasitica* var. *nicotianae* (FSU 746). *cycam* did not respond to all of these preparations which suggest that the isolated mutant has a general defect in responding to fungal signals with regard to $[Ca^{2+}]_{cyt}$ elevation. Interestingly, CYCAM mutant responded like WT to the CWE from another fungus, *Mortierella hyalina* (FSU 509).

The active component from Mh-CWE was separated first with a LC-18-DB column, 25cm x 4.60 mm ID (Suppelco), then with a Symmetry 250 × 2.1 mm column packed with 3 µm C18 stationary phase (Waters) and finally purified with an Acclaim C18 Column, 250 x 2.1 mm, 2.2 µm, (Dionex). Acetonitrile:water gradient was used as the mobile phase for 30 min run with a flow rate of 1 ml/min for HPLC and 0,3 ml/min for UPLC columns. Mh-CWE is purified as Mh-222 with m/z of 223.0599 (Figure 8).

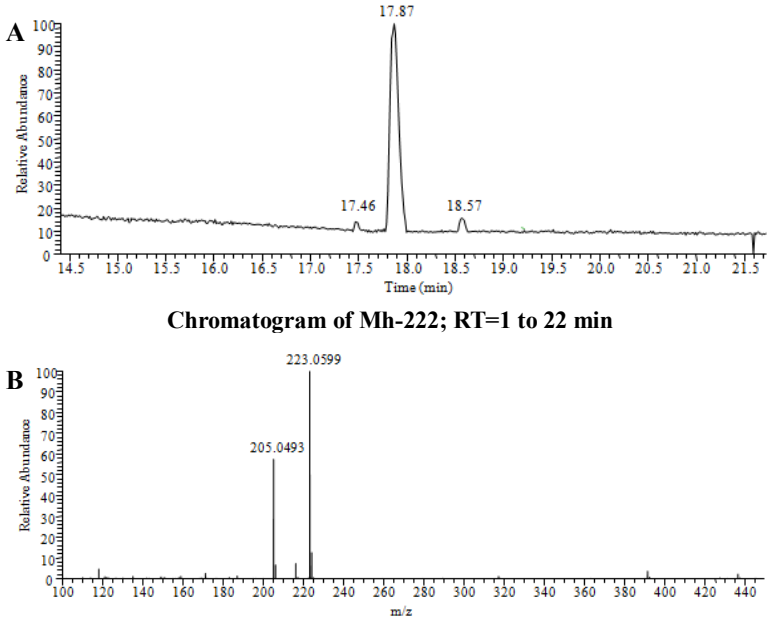


Figure 8. Purification of Mh-222 from Mh-CWE. Mh-222 was purified from the Mh-CWE as described earlier based on its property to induce $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis seedlings (WT and mutants). Mh-222 has an accurate molecular mass of m/z 223.0599. The chromatogram of Mh-222; RT=17.87 (A) and the high resolution LC-MS spectrum corresponding to RT=17.87 min (B) are shown.

Plants have evolved an elaborate system of generating $[Ca^{2+}]_{cyt}$ elevation which consists of a receptor, Ca^{2+} channels and pumps to control Ca^{2+} entry into cytoplasm, from apoplast and different cellular organelles that stores Ca^{2+} (Hetherington and Brownlee 2004; Reddy et al. 2011). Refractive nature of receptor studies shows that perception of different beneficial and pathogenic fungal signals, and subsequent $[Ca^{2+}]_{cyt}$ elevation are done by the same gene (*CYCAM*) except for the *A. brassicae*-toxin preparation, Mh-222 and chitin oligomers. Many well known receptors which perceive MAPM/PAMP have kinase domain, and modulate different developmental and stress related biological process in plants (Ranf *et al*. 2011, 2012; Wan *et al*. 2011, 2012; Vaid *et al*. 2013). Mutants with loss-function-of LYK1/CERK1 (Lysin motif-containing receptor-like kinase1/chitin elicitor receptor kinase1) resulted in the complete abolition of $[Ca^{2+}]_{cyt}$ elevation to different chitin oligomers and was highly susceptible to *A. brassisicola* (Wan *et al*. 2012; Tanaka *et al*. 2013). Total inhibition of different stimuli induced-$[Ca^{2+}]_{cyt}$ elevation by staurosporine, a kinase inhibitor suggests *CYCAM* contains a functional kinase domain. The above studies indicate that CYCAM may

be a receptor. Ca^{2+}-permeable channels present in the cell membrane or different membrane-bound organelles which store Ca^{2+} are the key entry points for Ca^{2+} into cytosol (Sanders *et al*. 2002). $[Ca^{2+}]_{cyt}$ elevation is attributed to Ca^{2+} influx through specific Ca^{2+} selective channels, both at the whole-cell and single channel level (c.f. Hetherington and Brownlee 2004). Ca^{2+}-permeable channels in Arabidopsis are encoded by 41 genes of the cyclic nucleotide gated channel (CNGC), glutamate receptor channel (GLR), and the two pore calcium channel (TPC) (c.f. Hetherington and Brownlee 2004). Lesion in Ca^{2+} channels and pumps impairs PAMP/MAMP induced-$[Ca^{2+}]_{cyt}$ elevation and affects the defense gene activation. In Pep/-PEPR (plant elicitor peptide/plant elicitor peptide receptor) system, Pep-induced $[Ca^{2+}]_{cyt}$ elevation is attributable to cGMP activation of a Ca^{2+} channel, CNGC2 and lesion in PEPR significantly reduced $[Ca^{2+}]_{cyt}$ elevation induced by Pep and were more susceptible to bacterial infection (Qi *et al*. 2010; Ma *et al*. 2012). Similarly, other well studied flg22/FLS2 (flagellin 22/Flagellin-Sensitive 2), elf18/EFR (elongation factor Tu/Elongation factor Tu receptor) and elf18/BAK1 (Brassinosteroid receptor 1-Associated Kinase 1) systems, lesions in Ca^{2+} channels also affected $[Ca^{2+}]_{cyt}$ elevation and the performance of mutants to biotic and abiotic stress (Chinchilla *et al*. 2009; Ranf *et al*. 2011, 2012). But in the above systems, lesions in Ca^{2+} channels did not completely inhibit $[Ca^{2+}]_{cyt}$ elevation. We propose that *CYCAM* is a general but very important gene to induce $[Ca^{2+}]_{cyt}$ elevation in response to signaling molecules from the beneficial endosymbionts and pathogenic fungi, and may be either a general receptor or a major Ca^{2+} channel or pump.

5.6 CYCAM is essential for tolerance to abiotic and biotic stress

$[Ca^{2+}]_{cyt}$ elevation is pivotal for the rapid response of plants to biotic and abiotic stress (Lecoureux *et al*. 2006; Dodd *et al*. 2010). *cycam* was more sensitive to salt, water, drought and oxidative stress. The importance of $[Ca^{2+}]_{cyt}$ is well demonstrated in water-, drought-, salt- (Knight *et al*. 1997) and oxidative- (Klüsener *et al*. 2002) stress signals in Arabidopsis. It is well-known that both ABA- and H_2O_2-induced $[Ca^{2+}]_{cyt}$ elevations in guard cells regulate the opening and closing of stomata which in turn control the absorption of water and nutrients (Schroeder *et al*. 2001; Klüsener *et al*. 2002). The mutants were more sensitive to paraquat which induces endogenous ROS and 3-aminotriazole which induces endogenous H_2O_2 levels in tissue. Therefore, we propose that CYCAM is also involved in abiotic stress responses. The mutants were also hypersusceptible to *A. brassicae* infection and more sensitive to *A. brassicae*-toxin preparation. *A. brassicae* produces host specific toxins which can also mimic

the symptoms as the fungus does (Walton 1996; Moebius and Hertweck 2009; Pedras and Khallaf 2012). High percentage disease index (PDI) and low biomass in *cycam* with *A. brassicae* infection clearly shows that there is break down of defense and CYCAM is very crucial for conferring resistance against *A. brassicae* infection (Figure 7). Moreover, Pi-504- and Ab-CWE-, EPM-, and EPS- induced signaling cascades may be repressing the Ab-toxin-induced signaling cascades involved in enhanced susceptibility to *A. brassicae* (Figure 7). PAMP-induced $[Ca^{2+}]_{cyt}$ changes are one of the early steps in pathogen perception and are attributed to activate the plant's surveillance system against the attempted pathogen invasion by modulating an array of defense responses in plants (Lecourieux *et al*. 2006; Dodd *et al*. 2010).

5.7 Hypersusceptibility of *cycam* to *A. brassicae* is correlated to high ABA and low (+)-7-*iso*-JA-Ile levels

ABA, SA and JA play key roles in mediating disease response to different fungal pathogens (c.f. Mauch-Mani and Mauch 2005; Bari and Jones 2009; Vlot *et al*. 2009). Studies with bitrophic, hemibiotrophic and necrotrophic pathogens on ABA mutants demonstrated that ABA is a negative regulator of plant defense (Mauch-Mani and Mauch 2005; Fan *et al*. 2009; Sanchez-Vallet *et al*. 2012). The hypersusceptibility of *cycam* to *A. brassicae* and its toxin is positively correlated to higher expression of major ABA biosynthesis genes (*AtBG1*, *NCED3* and *TOC1*) and higher ABA levels (Figure 7). The ABA levels were already higher in *cycam* even when not exposed to stress, and they were more sensitive to the exogenous ABA application compared to WT. Therefore, elevated ABA in the mutant may be one reason for its hypersusceptibility to *A. brassicae* infection. *A.brassicae* infection also induced SA and SA-responsive genes, e.g. *PRI* and *NPR1* in *mutant* and WT seedlings, and these effects were stronger in the mutant which demonstrates the involvement of CYCAM in the SA response. SA has both negative and positive roles in plant defense against different fungal and bacterial pathogens (Vlot *et al*. 2009; Zhao *et al*. 2013). A phospholipase Dβ1 (pldβ1) mutant and mutants impaired in phosphatidic acid (PA) biosynthesis were more susceptible to *B. cinerea* infection compared to the WT, and this was positively correlated to a higher SA level in the infected mutant plants (Zhao *et al*. 2013), similar to our observations with *cycam*. PLDβ1 binds Ca^{2+}, hydrolyzes phospholipids to generate PA which is involved in hormone signaling and disease resistance (Camehl *et al*. 2011; Zhao *et al*. 2013). Therefore CYCAM-mediated $[Ca^{2+}]_{cyt}$ elevation may effect *PLDβ1* expression and PLDβ1 activation.

JA has a critical role in both resistance and susceptibility against bacterial and fungal pathogens (Thomma *et al*. 1998; Bari and Jones 2009; Egusa *et al*. 2009; Pieterse *et al*. 2012). *A. brassicae* infection induced a JA burst in *cycam* and WT eventhough its precursor, cis-OPDA (cis-12-oxo-phytodienoic acid), did not show a substantial increase. The JA burst was much higher in *cycam* compared to WT which is positively correlated with the higher expression levels of *MYC2*, *VSP2*, *JAZ1*, *Thi2.1* and *PDF1.2*. Upregulation of a marker gene of the MYC branch (*VSP2*) and of the ERF branch (*PDF1.2*) in *cycam* suggests that both branches of the JA pathway are involved in the higher susceptibility to *A. brassicae* infections. Necrotrophic fungal infection increases JA levels in Arabidopsis (Egusa *et al*. 2009; Su'udi *et al*. 2011). However, (+)-7-*iso*-jasmonoyl-1-isoleucine ((+)-7-*iso*-JA-Ile), the biologically active form of JA (Bednarek *et al*. 2009, 2011; Fonseca *et al*. 2009) was induced stronger in WT compared to *cycam*, and is positively correlated to the enhanced resistance in WT (Figure 7). A positive role of JA in defense response was also reported in different pathosystems (Zhao *et al*. 2013). Loss of function of CYCAM might impair the activity of enzymes which convert JA to (+)-7-*iso*-JA-Ile in the cytosol (Fonseca *et al*. 2009). The enhanced level of (+)-7-*iso*-JA-Ile in WT is positively correlated to the enhanced tolerance to *A. brassicae* infection in WT. Therefore, loss-of-function of CYCAM not only results in high induction of ABA, but also lower induction of (+)-7-*iso*-JA-Ile which makes *cycam* hypersusceptible to *A. brassicae* infections.

5.8 Hypersusceptibility of *cycam* to *A. brassicae* is positively correlated to ROS amplification

Phytopathogens and their PAMPs induce ROS and H_2O_2 as one of the earliest defense responses (Mittler *et al*. 2004; Lecourieux *et al*. 2006; O'Brien *et al*. 2012). The endosymbionts AMF and *P. indica*, and their bMAMPs did not induce ROS (Navazio *et al*. 2007; Vadassery *et al*. 2009a). We could show that Pi-504 and *P. indica* did not induce, whereas *A. brassicae* infection and Ab-toxin treatment induced ROS and H_2O_2 in both WT and *cycam*, but the induction was much higher in *cycam* compared to WT. Different ROS and H_2O_2 marker genes e.g. *RRTF1*, *bHLH*, zinc finger CCCH, *AP2/EREBP*, *DSR*, and *OMT*, *OXI1* and *DIN11* (Rentel *et al*. 2004; Mehterov *et al*. 2012) were upregulated more in the mutant compared to WT. Furthermore, different ROS scavenging enzymes such as FSD1, CSD2, APX1, MDAR2 and DHAR5 (Mittler *et al*. 2004) were downregulated in the roots and shoots of *cycam*. Therefore, elevated H_2O_2/ROS levels together with low ROS scavenging

enzymes are responsible for the ROS burst and subsequently, the higher susceptibility to *A. brassicae* infection in the mutant (Figure 7). ROS at low concentration functions as signaling molecule for plant defense, but at higher concentration they are cytotoxic and predispose the cells to higher levels of infection (Heller and Tudzynski 2011; Mittler *et al*. 2011). ROS play a pivotal role in successful recognition of pathogen infection and activation of basal defense reactions (c.f. Torres 2010; Heller and Tudzynski 2011) and different necrotrophic fungi use these basal defense to facilitate their infection and successive colonization in their hosts by producing effectors/toxins (Gechev *et al*. 2004; Govrin *et al*. 2006; Su'udi *et al*. 2011; Zhao *et al*. 2013). Induction of ROS/H_2O_2 in biotrophic and hemibiotrophic interactions restrict further growth and colonization of fungi by activating secondary levels of defense in Arabidopsis (Heller and Tudzynski 2011; Lenz *et al*. 2011). Therefore, ROS/H_2O_2 induced during different plant-microbes interactions has different biological functions. H_2O_2/ROS burst to the cytotoxic level in *cycam* is correlated to the loss of function of $[Ca^{2+}]_{cyt}$ elevation induced by Pi-504 and *A. brassicae*-CWE (Figure 7).

5.9 Differential regulation of redox responsive transcription factor1 (RRTF1) in beneficial and pathogenic interactions in *A. thaliana*

Redox regulated transcription factor1 (RRTF1) is an evolutionarily conserved APETALA2/-ethylene response transcription factor (AP2/ERF) in angiosperms and is present in the major crop plants (Nakano *et al*. 2006). Khandelwal *et al*. (2008) reported *RRTF1* for the first time as the major component of a core redox signaling network that includes *EDS1*, *WRKY33* and different transcription factors with Zn-finger type and AP2 domains. *RRTF1* is induced by different biotic and abiotic stress (Toufighi *et al*. 2005; Khandelwal *et al*. 2008; Mehterov *et al*. 2012; Kerchev *et al*. 2013), whereas biotrophic and hemibiotrophic fungi repress *RRTF1* expression (Pandey *et al*. 2010). Mehterov *et al*. (2012) reported that *RRTF1* is the most important and sensitive ROS marker gene which is expressed in very high level in response to higher endogenous levels of H_2O_2 and ROS in Arabidopsis. We propose that RRTF1 could be a main hub to amplify ROS formation in plant cells. The necrotrophic pathogenic fungus *A. brassicae* stimulates *RRTF1* expression which amplifies ROS/H_2O_2 formation in the infected tissues and predisposes to rapid development of disease symptoms in the overexpressor lines. In contrast, the root endophyte *P. indica* represses *RRTF1*, thereby ROS/H_2O_2 burst in the colonized roots locally and also systemically in shoots which helped the plants to resist or tolerate biotic and abiotic stress (Figure 7). Moreover, *P. indica* also activated ROS

scavenging enzymes in different cellular compartments, e.g. APX1, MDAR2 and DHAR5 in cytoplasm, and FSD1, CSD2 and DHAR5 in chloroplast, which helps the plant to remove excess ROS. We propose that *P. indica* represses ROS/H_2O_2 accumulation by downregulating *RRTF1* and upregulating ROS scavenging enzymes, whereas *A. brassicae* activates ROS/H_2O_2 burst by inducing *RRTF1* and repressing ROS scavenging enzymes (Figure 7). In Arabidopis, *RbohD* and *RbohF* are important for plant defense (Torres *et al*. 2002; Marino *et al*. 2012) and are synergistically activated by phosphorylation and binding of Ca^{2+} to their hydrophilic N-terminal regions (Ogasawara *et al*. 2008). $[Ca^{2+}]_{cyt}$ elevations have been reported both upstream and downstream of ROS production indicating complex spatiotemporal Ca^{2+} and ROS elevation mechanisms (Kawano and Muto 2000; Blume *et al*. 2000). Different ROS inhibitors did not inhibit the $[Ca^{2+}]_{cyt}$ elevations induced by Pi-504 and *A. brassicae*-toxin which indicate that Pi-504- and *A. brassicae*-toxin preparation-induced $[Ca^{2+}]_{cyt}$ elevations are upstream of ROS induction. Crossing of pMAQ2 with *rrtf1* which has reduced level of ROS and its overexpressors (*oe18* and *oe20*) which accumulate more ROS to the cytotoxic level (Matsuo *et al*. 2013 revision), did not show any difference in $[Ca^{2+}]_{cyt}$ elevations induced by Pi-504 and *A. brassicae*-toxin in the F_3 progenies of *rrtf1*xpMAQ2, *oe18x*pMAQ2 and *oe20*xpMAQ2 lines. These results further reinstate that Pi-504- and *A. brassicae*-toxin-induced $[Ca^{2+}]_{cyt}$ elevations are upstream RRTF1 which amplifies ROS (Figure 7).

5.10. Glucosinolates restrict *P. indica* colonization in root and *A. brassicae* infection in shoot

Indolic and aliphatic glucosinolates and camalexin are important sulphur containing secondary metabolites involved in plant defense against phytopathogens (Halkier and Gershenzon 2006; Bednarek *et al*. 2009). These secondary metabolites are either constitutively present or induced in response to fungal invasion (Stotz *et al*. 2011; Su'udi *et al*. 2011; Foley *et al*. 2013; Wang *et al*. 2013). We demonstrate that *CYP79B2*, *CYP79B3* and *PAD3* involved in the camalexin and iGLS are important for the *P. indica*-mediated growth promotion in *A. thaliana*. The *cyp79B2 cyp79B3* double mutant, which lacks iGLS and camalexin, did not show growth promotion to *P. indica* in short- and long-term experimental conditions, whereas growth of the *pad3*, which does not synthesize camalexin, is not promoted by *P. indica* under long term interaction in soil even though it responded partially under short term experimental condition in Petri dishes (Nongbri et al. 2012). Therefore,

IAOx-derived compounds are crucial for establishing a beneficial interaction between *P. indica* and *A. thaliana* during the early and late stages of endosymbiosis.

The role of aGLS were well established in plant defense against herbivory and phytopathogens (Beekwilder et al. 2008; Stotz et al. 2011). Our studies with *myb28 myb29*, which lacks aGLS showed that this double knockout mutant responds to *P. indica*-mediated growth promotion like WT and does not play significant role in the beneficial endosymbiosis (Nongbri 2012). However, both *myb28 myb29* which does not have aGLS and the *cycam* which does not induce aGLS were hypersusceptible to *A. brassicae* infection. The induction of aliphatic glucosinolates, for example 5MSOP and 7MSOH were significantly high in WT compared to *cycam*. Moreover the mRNA levels of *MYB28*, *MYB29* and *BCAT4* (Halkier and Gershenzon 2006) which are involved in the aGLS biosynthesis, were also upregulated in WT and not in *cycam*, which reinstates that the induction of aGLS confers resistance to the fungal infection and is also regulated by $[Ca^{2+}]_{cyt}$ elevation. Therefore, we postulate that aGLS has a vital role as a positive modulator of plant defense against *A. brassicae* infection (Figure 7).

6. Summary

The beneficial interaction between the root-colonizing *Piriformospora indica* and *Arabidopsis thaliana* results in growth promotion, enhanced seed production and increased tolerance to biotic and abiotic stress. Perception of beneficial microbes and pathogens, and their associated molecular patterns (bMAMP, PAMP) leads to changes in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$). The model plant *Arabidopsis thaliana*, expressing the bioluminescent Ca^{2+} reporter apoaequorin in the cytosol, was used to elucidate the role of cytosolic Ca^{2+} signaling in the beneficial (*A. thaliana-P. indica*) and pathogenic (*A. thaliana-Alternaria brassicae*) interactions.

The biologically active component from the *P. indica*-cell wall extract (Pi-CWE) which induces $[Ca^{2+}]_{cyt}$ elevation in roots and stimulates the growth of *Arabidopsis* and tobacco seedlings, was purified as a trisaccharide ($C_{18}H_{32}O_{16}$) with *m/z* of 505.1748 (Pi-504).

Large scale screening of EMS mutagenised aequorin populations resulted in the isolation of *Pi-CYCAM* mutants which do not induce $[Ca^{2+}]_{cyt}$ elevation in response to Pi-504. The Pi-504- and the fungus- induced growth promotion is linked to enhanced nitrogen metabolism and phosphate uptake, and increased photosynthetic efficiency.

Independent screening of EMS mutants with an *A. brassicae*-cell wall extract (Ab-CWE) resulted in the isolation of *Ab-CYCAM* mutants which do not respond to the Ab-CWE, exudate preparations from mycelium (EPM) and germinating spores (EPS). However, $[Ca^{2+}]_{cyt}$ elevation was observed in response to Ab-toxin preparations. Crossing of *pi-cycam* and *ab-cycam* showed that the mutations are allelic. Initial mapping experiments have demonstrated that the mutated gene (*CYCAM*) is a recessive qualitative trait and located on chromosome 1.

The *CYCAM* mutants do not respond to exudate preparations from another endophytic beneficial fungus *Acremonium alternatum*, and pathogenic fungi e.g. *Rhizoctonia solani* and *Fusarium solani*; and the oomycete *Phytophthora parasitica* var. *nicotianae*, but it responded normally to a cell wall preparation from *Mortierella hyalina* (Mh-CWE). The active component in Mh-CWE was purified as Mh-222 with *m/z* of 223.0599. Different beneficial and pathogenic signals use the same gene to induce $[Ca^{2+}]_{cyt}$ elevation (*CYCAM*) which is very important to initiate early molecular events in beneficial and pathogenic interactions.

The *cycam* mutant is highly sensitive to abiotic (salt, water, drought and oxidative stress) and biotic (*A. brassicae* infection) stress. The mutant contains low levels of aliphatic

glucosinolates (aGLS) and the bioactive (+)-7-*iso*-jasmonoyl-1-isoleucine ((+)-7-*iso*-JA-Ile), increased level of abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and reactive oxygen species (ROS) upon *A. brassicae* infection. We propose that *cycam* is defective in a gene that is required for $[Ca^{2+}]_{cyt}$ elevation to establish biotic and abiotic stress responses.

The evolutionarily conserved redox responsive transcription factor1 (RRTF1) controls ROS production. *A. brassicae* infection stimulates *RRTF1* and represses ROS scavenging genes, while *P. indica* represses *RRTF1* and activates ROS scavenging enzymes. Genetic and pharmacological studies show that Pi-504-, Ab-CWE/EPM/EPS- and Ab-toxin-induced $[Ca^{2+}]_{cyt}$ elevations are upstream of RRTF1.

Camalexin and indolic glucosinolates (iGLS) are important for the beneficial interaction between *P. indica* and *A. thaliana*. These compounds restrict root colonization during different phases of the interaction. Furthermore, *P. indica*-derived auxin is not required for the growth response in Arabidopsis and Chinese cabbage. While auxin plays only a minor role in the *P. indica-A. thaliana* interaction, *P. indica*-induced growth promotion in Chinese cabbage is strongly dependent on auxin. We could show that the elevated auxin level in *P. indica*-colonized Chinese cabbage roots is of plant origin.

7. Zusammenfassung

Die vorteilhafte Interaktion zwischen dem Wurzel-kolonisierenden Pilz *Piriformospora indica* und *Arabidopsis thaliana* bewirkt Wachstumssteigerung, verstärkte Samenproduktion und erhöhte Toleranz gegenüber biotischem und abiotischem Stress. Die Perzeption von vorteilhaften Mikroben und pathogenen Pilzen und ihren assoziierten molekularen Strukturen (bMAMP, PAMP) führt zu Veränderungen in der cytosolischen Kalziumkonzentration, ($[Ca^{2+}]_{cyt}$). Die Modellpflanze *A. thaliana*, die den biolumineszenten Ca^{2+}-Reporter Apoaequorin im Cytosol exprimiert, wurde zur Untersuchung der Rolle von $[Ca^{2+}]_{cyt}$ in vorteilhaften (*A. thaliana-P. indica*) und pathogenen (*A. thaliana-Alternaria brassicae*) Interaktionen verwendet.

Die biologisch aktive Verbindung von *P. indica*-Zellwandextrakten (Pi-CWE), die eine Erhöhung der $[Ca^{2+}]_{cyt}$ in Wurzeln induziert und das Wachstum von *Arabidopsis*- und Tabak-Keimlingen fördert, wurde als ein Trisaccharid ($C_{18}H_{32}O_{16}$) mit einem *m/z*-Verhältnis von 505.1748 (Pi-504) gereinigt.

Durchsuchungen von EMS-mutagenisierten Aequorin-Populationen in großem Maßstab führten zur Isolierung von *Pi-CYCAM*-Mutanten (*cytosolic calcium mutants*), die auf Applikation von Pi-504 nicht mit einer Erhöhung von $[Ca^{2+}]_{cyt}$ reagieren. Sowohl die von Pi-504 als auch die durch den Pilz induzierte Wachstumssteigerung ist verknüpft mit einem erhöhten Stickstoffmetabolismus, erhöhter Phosphataufnahme und gesteigerter photosynthetischer Effizienz.

Unabhängiges Durchsuchen von EMS-Mutantenpopulationen mit dem Zellwandextrakt des pathogenen Pilzes *A. brassicae* führte zur Isolation von *Ab-CYCAM*-Mutanten, die nicht auf Ab-CWE reagieren. Ausserdem gibt es keine Erhöhung des $[Ca^{2+}]_{cyt}$ bei Exsudatpräparationen von Myzel (EPM) und von keimenden Sporen (EPS), aber auf das Ab-Toxin. Die Kreuzung von *pi-cycam* und *ab-cycam* zeigte, dass es sich um Allele handelt. Das mutierte Gen *CYCAM* ist ein rezessives, qualitatives Erbmerkmal und wurde auf Chromosom 1 lokalisiert.

CYCAM-Mutanten reagieren nicht auf das Exsudat anderen endophytischen Pilzes mit vorteilhafter Interaktion, *Acremonium alternatum*, und anderer pathogener Pilze wie z. B. *Rhizoctonia solani* und *Fusarium solani*; und dem Oomyzeten *Phytophthora parasitica* var. *nicotianae*. Sie reagieren jedoch auf Zellwandextrakte von *Mortierella hyalina* (Mh-CWE). Die aktive Komponente in Mh-CWE wurde als Mh-222 mit einem *m/z*-Verhältnis von

223.0599 aufgereinigt. Verschiedene vorteilhafte und pathogene Signale nutzen zur Erhöhung von $[Ca^{2+}]_{cyt}$ das gleiche Gen (*CYCAM*), welches frühe molekulare Vorgänge in vorteilhaften und pathogenen Interaktionen initiiert.

Die *CYCAM*-Mutante ist hoch sensitiv gegenüber abiotischen (Salz, Wasser, Trockenheit, oxidativer Stress) und biotischen (*A. brassicae*-Infektion und *A. brassicae*-Toxin) Stressfaktoren. Die Hypersuszeptibilität der *CYCAM*-Mutante gegenüber einer Infektion mit *A. brassicae* ist mit einem niedrigen Niveau von aliphatischen Glucosinolaten (aGLS) und bioaktivem (+)-7-*iso*-Jasmonoyl-1-Isoleucin ((+)-7-*iso*-JA-Ile) und einem höheren Niveau von Abscisinsäure (ABA), Salicylsäure (SA), Jasmonsäure (JA) und reaktiven Sauerstoffspezies (ROS) korreliert. Wir denken, dass *cycam* in einem Gen defekt ist, das für die Erhöhung der $[Ca^{2+}]_{cyt}$ notwendig ist, um auf biotischen und abiotischen Stress zu reagieren.

Der evolutionär konservierte Redox-responsive Transkriptionsfactor1 (*RRTF1*) reguliert die ROS Produktion. Infektion durch *A. brassicae* stimuliert *RRTF1* und reprimiert ROS-abbauende Genprodukte, während *P. indica* die *RRTF1* Expression reprimiert und ROS-abbauende Enzyme stimuliert. Genetische und pharmakologische Untersuchungen zeigen, dass durch Pi-504, Ab-CWE/EPM/EPS und Ab-Toxin induzierte $[Ca^{2+}]_{cyt}$-Erhöhungen vor der ROS-Verstärkung in *A. thaliana* stattfinden.

Camalexin sowie Indolglucosinolate (iGLS) sind sehr bedeutsam für die Etablierung der endosymbiotischen, vorteilhaften Interaktion von *P. indica* mit *A. thaliana*. Diese Komponenten kontrollieren die Wurzelkolonisierung während verschiedener Phasen der Interaktion. Auxin spielt nur eine untergeordnete Rolle bei der Wachstumsstimulierung in Arabidopsis, wohingegen die durch *P. indica* induzierte Wachstumsstimulierung in Chinakohl stark von Auxin abhängig. Wir konnten zeigen, dass das Auxin in *P. indica* kolonisiertem Chinakohlwurzeln pflanzlichen Ursprungs ist.

8. Literature cited

Akiyama, K., Matsuzaki, K. and Hayashi, H. (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature*, **435**, 824-827.

Bains, P.S. and Tewari, J.P. (1987) Purification, chemical characterization and host specificity of the toxin produced by *Alternaria brassicae*. *Physiol Mol Plant Pathol*, **30**, 259-271.

Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.H., Schäfer, P., Schwarczinger, I., Zuccaro, A. and Skoczowski, A. (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol*, **180**, 501-510.

Barazani, O., von Dahl, C.C. and Baldwin, I.T. (2007) *Sebacina vermifera* promotes the growth and fitness of *Nicotiana attenuata* by inhibiting ethylene signaling. *Plant Physiol*, **144**, 1223-1232.

Bari, R. and Jones, J.D.G. (2009) Role of plant hormones in plant defence responses. *Plant Mol Biol*, **69**, 473-488.

Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina. A. and Schulze-Lefert, P. (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science*, **323**, 101-106.

Bednarek, P., Piślewska-Bednarek, M., van Themaat, E.V.L., Maddula, R.K., Svatoš, A. and Schulze-Lefert, P. (2011) Conservation and clade-specific diversification of pathogen inducible tryptophan and indole glucosinolate metabolism in *Arabidopsis thaliana* relatives. *New Phytol*, **192**, 713-726.

Beekwilder, J., van Leeuwen, W., van Dam, N.M., Bertossi, M., Grandi, V., Mizzi, L., Soloviev, M., Szabados, L., Molthoff, J.W., Schipper, B., Verbocht, H., de Vos, R.C., Morandini, P., Aarts, M.G. and Bovy, A. (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in Arabidopsis. *PLoS One*, **3**, e2068.

Bell, C.J. and Ecker, J.R. (1994) Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. *Genomics*, **19**, 137-144.

Blume, B., Nürnberger, T., Nass, N. and Scheel, D. (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defence in parsley. *Plant Cell*, **12**, 1425-1440.

Bonfante, P. and Requena, N. (2011) Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. *Curr Opin Plant Biol*, **14**, 451-457.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J. and Oelmüller, R. (2010) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. *New Phytol*, **185**, 1062-1073.

Camehl, I., Drzewiecki, C., Vadassery, J., Shahollari, B., Sherameti, I., Forzani, C., Munnik, T., Hirt, H. and Oelmüller, R. (2011) The OXI1 kinase pathway mediates *Piriformospora indica*-induced growth promotion in Arabidopsis. *PLoS Pathog*, **7**, e1002051.

Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E., Maillet, F., Rosenberg, C., Cook, D., Gough, C. and Denarie, J. (2000) Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. *Plant Cell*, **12**, 1647-1665.

Cheng, S.H., Willmann, M.R., Chen, H.C. and Sheen, J. (2002) Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. *Plant Physiol*, **129**, 469-485.

Chinchilla, D., Shan, L., He, P., de Vries, S. and Kemmerling, B. (2009). One for all: the receptor-associated kinase BAK1. *Trends Plant Sci*, **14**, 535-541.

Contesto, C., Milesi, S., Mantelin, S., Zancarini, A., Desbrosses, G., Varoquaux, F., Bellini, C., Kowalczyk, M. and Touraine, B. (2010) The auxin-signaling pathway is required for the lateral root response of Arabidopsis to the rhizobacterium *Phyllobacterium brassicacearum*. *Planta*, **232**, 1455-1470.

Deshmukh, S., Hückelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F. and Kogel, K.H. (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proc Natl Acad Sci USA*, **103**, 18450-18457.

Dodd, A.N., Kudla, J. and Sanders, D. (2010) The language of calcium signaling. *Annu Rev Plant Biol,* **61***, 593-620.*

Egusa, M., Ozawa, R., Takabayashi, J., Otani, H. and Kodama, M. (2009) The jasmonate signaling pathway in tomato regulates susceptibility to a toxin-dependent necrotrophic pathogen. *Planta*, **229**, 965-976.

Ehrhardt, D.W., Wais, R. and Long, S.R. (1996) Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. *Cell*, **85**, 673-681.

Fan, J., Hill, L., Crooks, C., Doerner, P. and Lamb, C. (2009) Abscisic acid has a key role in modulating diverse plant-pathogen interactions. *Plant Physiol*, **150**, 1750-1761.

Foley, R.C., Gleason, C.A., Anderson, J.P., Hamann, T. and Singh, K.B. (2013) Genetic and genomic analysis of *Rhizoctonia solani* interactions with Arabidopsis; evidence of resistance mediated through NADPH oxidases. *PLoS One*, **8**, e56814.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C. and Solano, R. (2009) (+)-7-*iso*-Jasmonoyl-l-isoleucine is the endogenous bioactive jasmonate. *Nat Chem Biol*, **5**, 344-350.

Franken, P. (2012) The plant strengthening root endophyte *Piriformospora indica*: potential application and the biology behind. *Appl Microbiol and Biotech*, **96**, 1455-1464.

Gechev, T.S., Gadjev, I.Z. and Hille, J. (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity

of programmed cell death in plants. *Cell Mol Life Sci*, **61**, 1185-1197.

Govrin, E.M., Rachmilevitch, S., Tiwari, B.S., Solomon, M. and Levine, A. (2006) An elicitor from *Botrytis cinerea* induces the hypersensitive response in *Arabidopsis thaliana* and other plants and promotes the gray mold disease. *Phytopathol*, **96**, 299-307.

Gutjahr, C., Novero, M., Guether, M., Montanari, O., Udvardi, M. and Bonfante P. (2009) Presymbiotic factors released by the arbuscular mycorrhizal fungus *Gigaspora margarita* induce starch accumulation in *Lotus japonicus* roots. *New Phytol*, **183**, 53-61.

Halkier, B.A. and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annu Rev Plant Biol*, **57**, 303-333.

Heller, J. and Tudzynski, P. (2011) Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. *Annu Rev Phytopathol*, **49**, 369-390.

Hetherington, A.M. and Brownlee, C. (2004) The generation of Ca^{2+} signals in plants. *Annu Rev Plant Biol*, **55**, 401-427.

Hilbert, M., Voll, M.L., Ding, Y., Hofmann, J., Sharma, M. and Zuccaro, A. (2012) Indole derivative production by the root endophyte *Piriformospora indica* is not required for growth promotion but for biotrophic colonization of barley roots. *New Phytol*, **196**, 520-534.

Hu, X., Neill, S.J., Yang, Y. and Cai, W. (2009) Fungal elicitor Pep-25 increases cytosolic calcium ions, H_2O_2 production and activates the octadecanoid pathway in *Arabidopsis thaliana*. *Planta*, **229**, 1201-1208.

Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., Kogel, K.H. and Schäfer, P. (2011) Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. *Plant Physiol*, **156**, 726-740.

Jäschke, D., Dugassa-Gobena, D., Karlovsky, P., Vidal, S. and Ludwig-Müller, J. (2010) Suppression of clubroot (*Plasmodiophora brassicae*) development in *Arabidopsis thaliana* by the endophytic fungus *Acremonium alternatum*. *Plant Pathol*, **59**, 100-111.

Johnson, J.M., Lee, Y.C., Camehl, I., Sun, C., Yeh, K.W. and Oelmüller, R. (2013a) *Piriformospora indica* promotes growth of chinese cabbage by manipulating auxin homeostasis: Role of auxin in *P. indica* symbioses. In. *Piriformospora indica*: *sebacinales and their biotechnological applications*; *Soil Biology*, **33**, 139-148. A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg Germany.

Johnson, J.M., Nongbri, P.L., Sherameti, I. and Oelmüller, R. (2011a) Calcium signaling and cytosolic calcium measurements in plants. *Endocyt Cell Res*, **21**, 64-76.

Johnson, J.M. and Oelmüller, R. (2009) Mutualism or parasitism: life in an unstable continuum. What can we learn from the mutualistic interaction between *Piriformospora indica* and *Arabidopsis thaliana*? *Endocyt Cell Res*, **19**, 81-110.

Johnson, J.M. and Oelmüller, R. (2013). Agony to Harmony-What decides? Calcium signaling in beneficial and pathogenic plant-fungus interactions - What can we learn from

the *Arabidopsis/Piriformospora indica* Symbiosis?. In. *Molecular Microbial Ecology of the Rhizosphere*, **2**, 833-850, First Edition. Edited by Frans J. de Bruijn. © 2013 Wiley-Blackwell. Published 2013 by John Wiley & Sons, Inc.

Johnson, J.M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Varma, A. and Oelmüller, R. (2011b) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation - A model system to study plant beneficial traits. *Endocyt Cell Res*, **21**, 101-113.

Johnson, J.M., Sherameti, I., Nongbri P.L., and Oelmüller, R. (2013b) Standardized conditions to study beneficial and nonbeneficial traits in the *Piriformospora indica*/ *Arabidopsis thaliana* interaction. In. *Piriformospora indica*: *sebacinales and their biotechnological applications*; *Soil Biology*, **33**, 325-343. A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg Germany.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323-329.

Kawano, T. and Muto, S. (2000) Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco cell suspension culture. *J Exp Bot*, **51**, 685-693.

Kerchev, P.I., Karpińska, B., Morris, J.A., Hussain, A., Verrall, S.R., Hedley, P.E., Fenton, B., Foyer, C.H. and Hancock, R.D. (2013) Vitamin C and the abscisic acid-insensitive 4 transcription factor are important determinants of aphid resistance in Arabidopsis. *Antioxid Redox Signal*, **18**, 2091-2105.

Khandelwal, A., Elvitigala, T., Ghosh, B. and Quatrano, R.S. (2008) Arabidopsis transcriptome reveals control circuits regulating redox homeostasis and the role of an AP2 transcription factor1. *Plant Physiol*, **148**, 2050-2058.

Kim, M.C., Panstruga, R., Elliott, C., Muller, J., Devoto, A., Yoon, H.W., Park, H.C., Cho, M.J. and Schulze-Lefert, P. (2002) Calmodulin interacts with MLO protein to regulate defence against mildew in barley. *Nature*, **416**, 447-451.

Klüsener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V. and Schroeder, J.I. (2002) Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. *Plant Physiol*, **130**, 2152-2163.

Kogel, K.H., Franken, P. and Hückelhoven, R. (2006) Endophyte or parasite-what decides? *Curr Opin Plant Biol*, **9**, 358-363.

Kosuta, S., Chabaud, M., Lougnon, G., Gough, C., Denarie, J., Barker, D.G. and Becard, G. (2003) A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots of *Medicago truncatula*. *Plant Physiol*, **131**, 952-962.

Kosuta, S., Hazledine, S., Sun, J., Miwa, H., Morris, R.J., Downie, J.A. and Oldroyd, G.E.D. (2008) Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. *Proc Natl Acad Sci USA*, **105**, 9823-9828.

Knight, H., Trewavas, A.J. and Knight, M.R. (1997) Calcium signalling in *Arabidopsis*

thaliana responding to drought and salinity. *Plant J*, **12**, 1067-1078.

Knight, M.R., Campbell, A.K., Smith, S.M. and Trewavas, A.J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature*, **352***,* 524-*526.*

Kudla, J., Batistic, O. and Hashimoto, K. (2010) Calcium signals: the lead currency of plant information processing. *Plant Cell*, **22**, 541-563.

Kumar, M., Yadav, V., Tuteja, N. and Johri, A.K. (2009) Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. *Microbiol*, **155**, 780-790.

Kumar, V., Sarma, M.V.R.K., Saharan, K., Srivastava, R., Kumar, L., Sahai, V., Bisaria, V.S. and Sharma, A.K. (2012) Effect of formulated root endophytic fungus *Piriformospora indica* and plant growth promoting rhizobacteria fluorescent pseudomonads R62 and R81 on *Vigna mungo*. *World J Microbiol Biotech*, **28**, 595-603.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R. and Pugin, A. (2002) Analysis and effects of cytosolic free calcium elevation in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell*, **14**, 2627-2641.

Lecourieux, D., Ranjeva, R. and Pugin, A. (2006) Calcium in plant defence-signalling pathways. *New Phytol*, **171**, 249-269.

Lee, Y.C., Johnson, J.M., Chien, C.T., Sun, C., Cai, D., Lou, B., Oelmüller, R. and Yeh, K.W. (2011) Growth promotion of Chinese cabbage and Arabidopsis by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin. *Mol Plant Microbe Interact*, **24**, 421-431.

Lee, J. and Rudd, J.J. (2002) Calcium-dependent protein kinases: versatile plant signalling components necessary for pathogen defence. *Trends in Plant Sci*, **7**, 97-98.

Lenz, H.D., Haller, E., Melzer, E., Kober, K., Wurster, K., Stahl, M., Bassham, D.C., Vierstra, R.D., Parker, J.E., Bautor, J., Molina, A., Escudero, V., Shindo, T., van der Hoorn, R.A.L., Gust, A.A. and Nürnberger, T. (2011) Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens. *Plant J*, **66**, 818-830.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S. and Schulze-Lefert, P. (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. *Science*, **310**, 1180-1183.

Ludwig-Müller, J. and Güther, M. (2007) Auxins as signals in arbuscular mycorrhiza formation. *Plant Signal Behav*, **2**, 194-196.

Lukowitz, W., Gillmor, C.S. and Scheible, W.R. (2000) Positional cloning in Arabidopsis: why it feels good to have a genome initiative working for you. *Plant Physiol*, **123**, 795-805.

Ma, Y., Walker, R.K., Zhao, Y. and Berkowitz, G.A. (2012) Linking ligand perception by PEPR pattern recognition receptors to cytosolic Ca^{2+} elevation and downstream immune signaling in plants. *Proc Natl Acad Sci USA*, **109**, 19852-19857.

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., Giraudet,

D., Formey, D., Martinez, E.A., Driguez, H., Becard, G. and Denarie, J. (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature*, **69**, 58-63.

Marino, D., Dunand, C., Puppo, A. and Pauly, N. (2012) A burst of plant NADPH oxidases. *Trends Plant Sci*, **17**, 9-15.

Matsuo, M., Johnson, J.M., Godfrey, R., Obokata, J., Böhmer, F.D. and Oelmüller, R. (2013) Redox responsive transcription factor1 amplifies the formation of reactive oxygen species in *Arabidopsis thaliana* shoots and roots. *Plant Cell*, under revision.

Mauch-Mani, B. and Mauch, F. (2005) The role of abscisic acid in plant-pathogen interactions. *Curr Opin Plant Biol*, **8**, 409-414.

Maxwell, K. and Johnson, G.N. (2000) Chlorophyll fluorescence - a practical guide. *J Exp Bot*, **51**, 659-668.

Mehterov, N., Balazadeh, S., Hille, J., Toneva, V., Müller-Röber, B. and Gechev, T. (2012) Oxidative stress provokes distinct transcriptional responses in the stress-tolerant *atr7* and stress-sensitive *loh2 Arabidopsis thaliana* mutants as revealed by multi-parallel quantitative real-time PCR analysis of ROS marker and antioxidant genes. *Plant Physiol Biochem*, **59**, 20-29.

Mithöfer, A., Ebel, J., Bhagwat, A.A., Boller, T. and Neuhaus-Url, G. (1999) Transgenic aequorin monitors cytosolic transients in soybean cells challenged with β-glucan or chitin elicitors. *Planta*, **207**, 566-574.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci*, **9**, 490-498.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V. and Van Breusegem, F. (2011) ROS signaling: the new wave? *Trends Plant Sci*, **16**, 300-309.

Moebius, N. and Hertweck, C. (2009) Fungal phytotoxins as mediators of virulence. *Curr Opin Plant Biol*, **12**, 390-398.

Molitor, A., Zajic, D., Voll, L.M., Pons-Kuhnemann, J., Samans, B., Kogel, K.H. and Waller, F. (2011) Barley leaf transcriptome and metabolite analysis reveals new aspects of compatibility and *Piriformospora indica*-mediated systemic induced resistance to powdery mildew. *Mol Plant Microb Interact*, **24**, 1427-1439.

Nakano, T., Suzuki, K., Fujimura, T. and Shinshi, H. (2006). Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol*, **140**, 411-432.

Navazio, L., Moscatiello, R., Genre, A., Novero, M., Baldan, B., Bonfante, P. and Mariani, P. (2007) A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. *Plant Physiol*, **144**, 673-681.

Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D. and Hancock, J.T. (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J Exp Bot*, **53**, 1237-1247.

Nongbri, P.L. (2012) The role of glucosinolates in the Arabidopsis/*Piriformospora indica* interaction. Dissertation, Friedrich-Schiller-Universität Jena Germany.

Nongbri, P.L., Johnson, J.M., Sherameti I., Glawischnig E., Halkier B.A., and Oelmüller, R. (2012) Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*. *Mol Plant Microbe Interact*, **25**, 1186-1197.

Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W., Hahlbrock, K. and Scheel D. (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. *Cell*, **78**, 449-460.

O'Brien, J.A., Daudi, A., Butt, V.S. and Bolwell, G.P. (2012) Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta*, **236**, 765-779.

Oelmüller, R., Peškan-Berghöfer, T., Shahollari, B., Trebicka, A., Sherameti, I. and Varma, A. (2005) MATH domain proteins represent a novel protein family in *Arabidopsis thaliana*, and at least one member is modified in roots during the course of a plant-microbe interaction. *Physiol Plant*, **124**, 152-166.

Oelmüller, R., Sherameti, I., Tripathi, S. and Varma, A. (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis*, **49**, 1-17.

Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., Hishinuma, H., Senzaki, E., Yamagoe, S., Nagata, K., Nara, M., Suzuki, K., Tanokura, M. and Kuchitsu, K. (2008) Synergistic activation of the Arabidopsis NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. *J Biol Chem*, **283**, 8885-8892.

Oldroyd, G.E. (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat Rev Microbiol*, **11**, 252-263.

Oldroyd, G.E., Murray, J.D., Poole, P.S. and Downie, J.A. (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet,* **45**, 119-144.

Pandey, S.P., Roccaro, M., Schön, M., Logemann, E. and Somssich, I.E. (2010). Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. *Plant J*, **64**, 912-923.

Parniske, M. (2008) Arbuscular mycorrhizal: the mother of plant root endosymbioses. *Nat Rev Microbiol*, **6**, 763-775.

Pedersen, B.P., Kumar, H., Waight, A.B., Risenmay, A.J., Zygy Roe-Zurz, Z., Chau, B.H., Schlessinger, A., Bonomi, M., Harries, W., Andrej Sali, A., Johri, A.K. and Stroud, R.M. (2013) Crystal structure of a eukaryotic phosphate transporter. *Nature*, **496**, 533-536.

Perrine-Walker, F., Doumas, P., Lucas, M., Vaissayre, V., Beauchemin, N.J., Band, L.R., Chopard, J., Crabos, A., Conejero, G., Péret, B., King, J.R., Verdeil, J.L., Hocher, V., Franche, C., Bennett, M.J., Tisa, L.S. and Laplaze, L. (2010) Auxin carriers localization drives auxin accumulation in plant cells infected by Frankia in *Casuarina*

glauca actinorhizal nodules. *Plant Physiol*, **154**, 1372-1380.

Pedras, M.S.C. and Khallaf, I. (2012) Molecular interactions of the phytotoxins destruxin B and sirodesmin PL with crucifers and cereals: Metabolism and elicitation of plant defenses. *Phytochem*, **77**, 129-139.

Pieterse, C.M.J., van der Does, D., Zamioudis, C., Leon-Reyes, A. and van Wees, S.C.M. (2012) Hormonal modulation of plant immunity. *Annu Rev Cell Dev Biol*, **28**, 489-521.

Qi, Z., Verma, R., Gehring, C., Yamaghuchi, Y., Zhao, Y., Ryan, C.A. and Berkowitz, G.A. (2010) Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels. *Proc Natl Acad Sci USA*, **107**, 21193-21198.

Qiang, X., Weiss, M., Kogel, K.H. and Schafer, P. (2012a) *Piriformospora indica* a mutualistic basidiomycete with an exceptionally large plant host range. *Mol Plant Pathol*, **13**, 508-518.

Qiang, X.Y., Zechmann, B., Reitz, M.U., Kogel, K.H. and Schafer, P. (2012b) The mutualistic fungus *Piriformospora indica* colonizes Arabidopsis roots by inducing an endoplasmic reticulum stress-triggered caspase-dependent cell death. *Plant Cell*, **24**, 794-809.

Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J. and Scheel, D. (2011) Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J*, **68**, 100-113.

Ranf, S., Grimmera, J., Pöschl, Y., Pecher, P., Chinchilla, D., Scheel, D. and Lee, J. (2012) Defense-related calcium signaling mutants uncovered *via* a quantitative high-throughput screen in *Arabidopsis thaliana*. *Mol Plant*, **5**, 115-130.

Reddy, A.S., Ali, G.S., Celesnik, H. and Day, I.S. (2011) Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *Plant Cell*, **23**, 2010-2032.

Reddy, V.S. and Reddy, A.S.N. (2004) Proteomics of calcium-signaling components in plants. *Phytochem*, **65**, 1745-1776.

Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. and Knight, M.R. (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. *Nature*, **427**, 858-861.

Sánchez-Vallet, A., López, G., Ramos, B., Delgado-Cerezo, M., Riviere, M.-P., Liorente, F., Fernández, P.V., Miedes, E., Estevez, J.M., Grant, M. and Molina, A. (2012) Disruption of abscisic acid signalling constitutively activates Arabidopsis resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. *Plant Physiol*, **160**, 2109-2124.

Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell*, **14**, S401-417.

Schäfer, P., Pfiffi, S., Voll, L.M., Zajic, D., Chandler, P.M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U. and Kogel, K.H. (2009a) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic

association of barley roots with *Piriformospora indica*. *Plant J,* **59**, 461-474.

Schäfer, P., Pfiffi, S., Voll, L.M., Zajic, D., Chandler, P.M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U. and Kogel, K.H. (2009b) Phytohormones in plant root-*Piriformospora indica* mutualism. *Plant Signal Behav*, **4**, 669-671.

Schroeder, J.I., Kwak, J.M. and Allen, G.J. (2001) Guard cell abscisic acid signalling and engineering drought hardiness in plants. *Nature*, **410**, 327-330.

Serfling, A., Wirsel, S.G.R., Lind, V., Deising, H.B. (2007) Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions. *Phytopathol*, **97**, 523-531.

Shahollari, B., Vadassery, J., Varma, A. and Oelmüller, R. (2007) A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. *Plant J*, **50**, 1-13.

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R. (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor which binds to a conserved motif in their promoters. *J Biol Chem*, **280**, 2641-2647.

Sherameti, I., Tripathi, S., Varma, A., and Oelmüller, R. (2008a). The root colonizing endophyte *Pirifomospora indica* confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. *Mol. Plant Microbe Interact*, **21**, 799-807.

Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V.M., Nitz, I., Varma, A., Grundler, F.M. and Oelmüller, R. (2008b) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. *Plant J*, **54**, 428-439.

Singh, S. and Parniske, M. (2012) Activation of calcium- and calmodulin-dependent protein kinase (CCaMK), the central regulator of plant root endosymbiosis. *Curr Opin Plant Biol*, **15**, 444- 453.

Sirrenberg, A., Göbel, C., Grond, S., Czempinski, N., Ratzinger, A., Karlovsky, P., Santos, P., Feussner, I. and Pawlowski, K. (2007) *Piriformospora indica* affects plant growth by auxin production. *Physiol Plant*, **131**, 581-589.

Smith, S.E. and Smith, S.F. (2011) Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. *Annu Rev Plant Biol*, **62**, 227-250.

Sonderby, I.E., Burow, M., Rowe, H.C., Kliebenstein, D.J. and Halkier, B.A. (2010) A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in Arabidopsis. *Plant Physiol*, **153**, 348-363.

Stein, E., Molitor, A., Kogel, K.H. and Waller, F. (2008) Systemic resistance in Arabidopsis

conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol*, **49**, 1747-1751.

Stotz, H.U., Sawada, Y., Shimada, Y., Hirai, M.Y., Sasaki, E., Krischke, M., Brown, P.D., Saito, K. and Kamiya, Y. (2011) Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of Arabidopsis against *Sclerotinia sclerotiorum*. *Plant J*, **67**, 81-93.

Sun, C., Johnson, J.M., Cai, D., Sherameti, I., Oelmüller, R. and Lou, B. (2010) *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *J Plant Physiol*, **167**, 1009-1017.

Su'udi, M., Kim, M.G., Park, S.R., Hwang, D.J., Bae, S.C. and Ahn, I.P. (2011) Arabidopsis cell death in compatible and incompatible interactions with *Alternaria brassicicola*. *Mol Cells*, **31**, 593-601.

Takahashi, F., Mizoguchi, T., Yoshida, R., Ichimura, K. and Shinozaki, K. (2011) Calmodulin-dependent activation of MAP kinase for ROS homeostasis in Arabidopsis. *Mol Cell*, **41**, 649-660.

Tanaka, K,, Nguyen, C.T., Liang, Y., Cao, Y. and Stacey, G. (2013) Role of LysM receptors in chitin-triggered plant innate immunity. *Plant Signal Behav*, **8**, e22598.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci USA*, **95**, 15107-15111.

Torres, M.A. (2010) ROS in biotic interactions. *Physiol Plant*, **138**, 414-429.

Torres, M.A., Dangl, J.L. and Jones, J.D. (2002) Arabidopsis *gp91phox* homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defence response. *Proc Natl Acad Sci USA*, **99**, 517-522.

Toufighi, K., Brady, S., Austin, R., Ly, E. and Provart, N. (2005) The botany array resource: e-northerns, expression angling, and promoter analyses. *Plant J*, **43**, 153-163.

Vadassery, J., Ritter, C., Venus, Y., Camehl, I., Varma, A., Shahollari, B., Novák, O., Strnad, M., Ludwig-Müller, J. and Oelmüller, R. (2008) The role of auxins and cytokinins in the mutualistic interaction between Arabidopsis and *Piriformospora indica*. *Mol. Plant Microbe Interact*, **21**, 1371-1383.

Vadassery, J., Ranf, S., Drewiecki, C., Mithöfer, A., Mazars, C., Scheel, D., Lee, J. and Oelmüller R. (2009a) A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *Plant J*, **59**, 193-206.

Vadassery, J., Tripathi, S., Prasad, R., Varma, A. and Oelmüller, R. (2009b) Monodehydroascorbate reductase 2 and dehydroascorbate reductase 5 are crucial for a mutualistic interaction between *Piriformospora indica* and Arabidopsis. *J Plant Physiol*,

166, 1263-1274.

Vaid, N., Macovei, A. and Tuteja, N. (2013). Knights in action: Lectin receptor-like kinases in plant development and stress responses. *Mol Plant*, doi: 10.1093.

van Wees, S.C., Van der Ent, S. and Pieterse, C.M. (2008) Plant immune responses triggered by beneficial microbes. *Curr Opin Plant Biol*, **11**, 443-448.

Varma, A., Verma, S., Sudha, S., Sahay, N., Butehorn, B. and Franken, P. (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. *Appl Environ Microbiol*, **65**, 2741-2744.

Varma, A., Bakshi, M., Lou, B., Hartmann, A. and Oelmueller, R. (2012) *Piriformospora indica*: A novel plant growth-promoting mycorrhizal fungus. *Agric Res*, **1**, 117-131.

Venus, Y. and Oelmüller, R. (2013) Arabidopsis ROP1 and ROP6 influence germination time, root morphology, the formation of F-Actin bundles, and symbiotic fungal interactions. *Mol Plant*, **6**, 872-886.

Verma, **S., Varma**, **A., Rexer**, **K.-H., Hassel**, **A., Kost**, **G., Sarbhoy**, **A., Bisen**, **P., Bütehorn**, **B. and Franken**, **P.** (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia*, 90: 898-905.

Vlot, A.C., Dempsey, D.A. and Klessig, D.F. (2009) Salicylic acid, a multifaceted hormone to combat disease. *Annu Rev Phytopathol*, **47**, 177-206.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P. and Kogel, K.H. (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc Natl Acad Sci USA*, **102**, 13386-13391.

Walton, J. D. (1996) Host-selective toxins: Agents of compatibility. *Plant Cell*, **8**, 1723-1733.

Wan, J., Tanaka, K., Zhang, X.C., Son, G.H., Brechenmacher, L., Nguyen, T.H. and Stacey, G. (2012) LYK4, a lysin motif receptor-like kinase, is important for chitin signaling and plant innate immunity in Arabidopsis. *Plant Physiol*, **160**, 396-406.

Wang, Y., Bouwmeester, K., van de Mortel, J.E., Shan, W. and Govers, F. (2013) A novel Arabidopsis-oomycete pathosystem: differential interactions with *Phytophthora capsici* reveal a role for camalexin, indole glucosinolates and salicylic acid in defence. *Plant Cell Environ*, **36**, 1192-1203.

Weiß, M., Sýkorová, Z., Garnica, S., Riess, K., Martos, F., Krause, C., Oberwinkler, F., Bauer, R. and Redecker, D. (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. *PLoS One*, **6**, e16793.

Xu, H. and Heath, M.C. (1998) Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus. *Plant Cell*, **10**, 585-598.

Yadav, V., Kumar, M., Deep, D.K., Kumar, H., Sharma, R., Tripathy, T., Tuteja, N., Saxena, A.K. and Johri, A.K. (2010) A Phosphate transporter from a root endophytic

fungus *Piriformospora indica* plays a role in the phosphate transfer to the plants. *J Biol Chem*, **285**, 26532-26544.

Yang, T. and Poovaiah, B.W. (2002) A calmodulin-binding/CGCG box DNA-binding protein family involved in multiple signaling pathways in plants. *J Biol Chem*, **277**, 45049-45058.

Zarea, M.J., Hajinia, S., Karimi, N., Goltapeh, E.M., Rejali, F., Varma, A. (2012) Effect of *Piriformospora indica* and Azospirillum strains from saline or non-saline soil on mitigation of the effects of NaCl. *Soil Biol Biochem*, **45**, 139-146.

Zhao, J., Devaiah, S. P., Wang, C., Li, M., Welti, R. and Wang, X. (2013) Arabidopsis phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens. *New Phytol*, **199**, 228-240.

Zuccaro, A., Lahrmann, U., Güldener, U., Langen, G., Pfiffi, S., Biedenkopf, D., Wong, P., Samans, B., Grimm, C., Basiewicz, M., Murat, C., Martin, F. and Kogel, K.H. (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. *PLoS Pathog*, **7**, e1002290.

9. Appendix

9.1 Declaration of independent assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in the experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University Jena or to any other University.

…….....

Joy Michal Johnson

Jena, February 06th 2014.

9.2 Curriculum vitae

Name	: Joy Michal Johnson
Date of birth	: 25-05-1971
Place of birth	: Thiruvananthapuram, Kerala, India
Nationality	: Indian
Present address	: 806, Bau 15; Ernst Abbe Platz 5; D 07743, Jena Germany.
Permanent address	: TC 44/114, Beroma cottage, Karuppai Road, Valiathura Vallakkadavoo P.O., Thiruvananthapuram 695 008 Kerala. email: joymkau@gmail.com; joy.michal-johnson@uni-jena.de

Professional education

Apr 1993-June 1995	M.Sc. (Ag.) in Plant Pathology, **Tamil Nadu Agricultural University**, Coimbatore, Tamil Nadu., 9.54/10.00
Oct 1988-Jan 1993	B.Sc. (Agri.), **Kerala Agricultural University**., 8.90/10.00

Professional experience (teaching, research and extension)

Nov 2007 onwards	PhD Scholar, Institute of General Botany and Plant Physiology, **Friedrich-Schiller-Universität Jena**, Germany.
Dec 2003-Oct 2007	Assistant Professor (Senior Scale) of Plant Pathology, **Kerala Agricultural University,** Kerala, India.
Aug 1999-Dec 2003	Assistant Professor of Plant Pathology, **Kerala Agricultural University,** Kerala, India.
Dec 1997-Aug1999	Scientist (Plant Pathology), **Indian Institute of Horticultural Research**, ICAR, Govt of India.
Nov 1995-Dec 1997	Junior Scientist (Plant Pathology), **Rubber Research Institute of India**, Govt. of India.

Academic distinctions

Leistungsprämie from The Kanzler, FSU Jena (Letter dt. 19th October 2010).

Agricultural Research Service (ARS) - Indian Council of Agricultural Research (ICAR), New Delhi, Government of India in 1996.

First Rank - Senior Research Fellowship examination in Plant Pathology conducted by ICAR, Government of India in 1995.

First Rank - M.Sc.(Ag) in Plant Pathology from Tamil Nadu Agricultural University in 1993.

First Rank - Junior Research Fellowship examination in Plant Pathology, ICAR, Government of India in 1992.

9.3 Leistungsprämie

seit 1558

Friedrich-Schiller-Universität Jena

Friedrich-Schiller-Universität Jena · Postfach · D-07737 Jena

PERSÖNLICH

Herrn
Joy Michal Johnson

Biologisch-Pharmazeutische Fakultät
Institut für Allgemeine Botanik und
Pflanzenphysiologie

Der Kanzler

Fürstengraben 1
D-07743 Jena

Telefon: 0 36 41 · 93 10 50
Telefax: 0 36 41 · 93 10 52

E-Mail: kanzler@uni-jena.de

Jena, 19. OKT. 2010

Leistungsprämie

Sehr geehrter Herr Michal Johnson,

die Friedrich-Schiller-Universität Jena dankt Ihnen für Ihr hohes persönliches Engagement und Ihren Einsatz. In Anerkennung Ihrer besonderen Leistungen erhalten Sie eine einmalige Leistungsprämie in Höhe von

EUR 560,00 brutto,

die mit der Gehaltszahlung im Dezember ausgezahlt wird.

Für Ihre weitere Tätigkeit an der Friedrich-Schiller-Universität Jena wünsche ich Ihnen Freude und Erfolg.

Mit freundlichen Grüßen

Dr. Klaus Bartholmé

9.4 List of publications and presentations (last 5 year)

Publications

Johnson, J.M., Maddula, R., Reichelt, M., Lorenz, S., Mitsuhiro M., Godfrey, R., Vadassery, J., Nongbri, P.L., Böhmer, F.D., Gershenzon, J., Schneider, B., Svatos, A. and Oelmüller, R. (2013) Growth and Defense - Ca^{2+} signaling in unstable continuum: *Piriformopsora indica*-derived trisaccharide induces intracellular calcium elevation, promotes growth and confers resistance in *Arabidopsis thaliana*. (**in preparation**).

Johnson, J.M., Reichelt, M., Nongbri, P.L., Vadassery, J., Gershenzon, J. and Oelmüller, R. (2013). A cell wall extract and exudates from *Alternaria brassicae* induce intracellular calcium elevation and is crucial for the enhanced tolerance to biotic and abiotic stress in *Arabidopsis thaliana*. (**in preparation**).

Mitsuhiro M.,* Johnson, J.M.,* Godfrey, R., Obokata, J., Böhmer, F.D. and Oelmüller, R. (2013) Redox responsive transcription factor1 amplifies the formation of reactive oxygen species in *Arabidopsis thaliana* shoots and roots. *Plant Cell*, **under revision**. (* equal contribution).

Nongbri, P.L.,* Johnson, J.M.,* Sherameti, I., Glawischnig, E., Halkier, B.A. and Oelmüller, R. (2012) Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between Arabidopsis roots and the endophyte *Piriformospora indica*. *Mol Plant Microbe Interact*, **25**, 1186-1197. (* equal contribution)

Nongbri, P.L., Vahabi, K., Mrozinska, A., Seebald, E., Sun, C., Sherameti, I., Johnson, J.M. and Oelmüller, R. (2012) Balancing defense and growth-Analyses of the beneficial symbiosis between *Piriformospora indica* and *Arabidopsis thaliana*. *Symbiosis*, **58**, 17-28.

Johnson, J.M., Nongbri, P.L., Sherameti, I. and Oelmüller, R. (2011) Calcium signaling and cytosolic calcium measurements in plants. *Endocyt Cell Res*, **21**, 64-76.

Johnson, J.M., Sherameti, I., Ludwig, A., Nongbri, P.L., Sun, C., Varma, A. and Oelmüller, R. (2011) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* cocultivation - A model system to study plant beneficial traits. *Endocyt Cell Res*, **21**, 101-113.

Lee, Y.C.,* Johnson, J.M.,* Chien, C.T., Sun, C., Cai, D., Lou, B., Oelmüller, R. and Yeh, K.W. (2011) Growth promotion of Chinese cabbage and Arabidopsis by *Piriformospora indica* is not stimulated by mycelium-synthesized auxin. *Mol Plant Microbe Interact*, **24**, 421-431. (* equal contribution)

Vahabi, K., Johnson, J.M., Drzewiecki, C. and Oelmüller, R. (2011) Fungal staining tools to study the interaction between the beneficial endophyte *Piriformospora indica* with *Arabidopsis thaliana* roots. *Endocyt Cell Res*, **21**, 77-88.

Sherameti, I., Johnson, J.M., Nongbri, P.L. and Oelmüller, R. (2010) The central role of iron and calcium for plant/microbe interaction and shaping microbial communities in the soil. *Albanian J Agric Sci*, **9**, 1-24.

Sun, C., Johnson, J.M., Cai, D., Sherameti, I., Oelmüller, R. and Lou, B. (2010) *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *J Plant Physiol*, **167**, 1009-1017.

Johnson, J.M. and Oelmüller, R. (2009) Mutualism or parasitism: life in an unstable continuum. What can we learn from the mutualistic interaction between *Piriformospora indica* and *Arabidopsis thaliana*? *Endocyt Cell Res*, **19**, 81-110.

Book chapters

Camehl, I., Sherameti, I., Seebald, E., Johnson, J.M. and Oelmüller, R. (2013) Role of defense compounds in the beneficial interaction between *Arabidopsis thaliana* and *Piriformospora indica*. In. *Piriformospora indica - Sebacinales and their biotechnological applications (2013), Soil Biology*, **33**, 239-250. A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg Germany.

Johnson, J.M., Lee, Y.C., Camehl, I., Sun, C., Yeh, K.W. and Oelmüller, R. (2013) *Piriformospora indica* promotes growth of chinese cabbage by manipulating auxin homeostasis: Role of auxin in *P. indica* symbioses. In. *Piriformospora indica: sebacinales and their biotechnological applications*; *Soil Biology*, **33**, 139-148. A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg Germany.

Johnson, J.M. and Oelmüller, R. (2013). Agony to Harmony-What decides? Calcium signaling in beneficial and pathogenic plant–fungus interactions - What can we learn from the *Arabidopsis/Piriformospora indica* Symbiosis?. In. *Molecular Microbial Ecology of the Rhizosphere*, **2**, 833-850, First Edition. Edited by Frans J. de Bruijn. © 2013 Wiley-Blackwell. Published 2013 by John Wiley & Sons, Inc.

Johnson, J.M., Sherameti, I., Nongbri P.L., and Oelmüller, R. (2013) Standardized conditions to study beneficial and nonbeneficial traits in the *Piriformospora indica*/

Arabidopsis thaliana interaction. In. *Piriformospora indica*: *sebacinales and their biotechnological applications*; *Soil Biology*, **33**, 325-343. A. Varma et al. (eds.), © Springer-Verlag Berlin Heidelberg. Germany.

Varma A., Sherameti I., Tripathi S., Prasad R., Das A., Sharma M., Bakshi, M., Johnson J.M., Bhardwaj S., Arora M., Rastogi K., Agarwal A., Kharkwal A.C., Talukdar S., Bagde U.S., Bisaria V.S., Upadhyaya C.P., Won P.S., Chen Y., Ma J., Lou B., Adya A., Zhong L., Meghvanshi M.K., Gosal S.K., Srivastava R.B., Johri A.K., Cruz C. and Oelmüller R. (2012) The Symbiotic Fungus *Piriformospora indica*: Review. In. *Fungal Associations, The Mycota IX:* 231-254. B. Hock (Ed.), © Springer-Verlag Berlin Heidelberg Germany.

Oral presentations

Johnson, J.M. and Oelmüller, R. (2013) AGONY to HARMONY: *RRTF1* - a novel transcription factor controlling ROS production, and its modulation in pathogenic and beneficial fungal interactions in *Arabidopsis thaliana*. Mitteldeutsche Pflanzenphysiologie Tagung, Halle (Salle), Germany.

Johnson, J.M., Reichelt, M., Maddula, R., Lorenz, S., Nongbri, P,L., Svatos, A. Schneider, B., Gershenzon, J. and Oelmüller, R. (2012) Agony to Harmony - Ca^{2+} signaling in unstable continuum: Role of *Piriformospora indica*-induced intracellular calcium elevation in beneficial and pathogenic plant-fungus interactions in *Arabidopsis thaliana*. MICOM 2012, Jena, Germany.

Johnson, J.M. and Oelmüller, R. (2012) Role of *RRTF1* in beneficial and pathogenic fungal interactions in *Arabidopsis thaliana*. Mitteldeutschen Pflanzenphysiologen, Jena, Germany.

Johnson, J.M. and Oelmüller, R. (2011) Arabidopsis mutants impaired in cytoplasmic calcium elevation in response to fungal stimuli. Südostdeutche Pflanzenphysiologie Tagung, Leipzig, Germany.

Johnson, J.M., and Oelmüller, R. (2010) Calcium signal transduction in the beneficial interaction between Arabidopsis and *Piriformospora indica*. 8th Mitteldeutsches Plant Physiologists Meeting, Dresden, Germany.

Johnson, J.M., Seebald, I., Vadassery, J. and Oelmüller, R. (2010) *Piriformospora indica*-induced intracellular calcium elevation confers resistance against necrotrophic fungus in Arabidospsis. 11th International Colloquium on Endocytobiology and Symbiosis, Tromso, Norway.

Johnson, J.M., Seebald, I., Vadassery, J. and Oelmüller, R. (2009) *Arabidopsis* mutants defective in *Piriformospora indica* induced intracellular calcium elevation. Mitteldeutsches Treffen der Pflanzenphysiologen, Halle (Saale), Germany.

Johnson, J.M., Seebald, I., Vadassery, J. and Oelmüller, R. (2009) *Arabidopsis* mutants defective in *Piriformospora indica* induced intracellular calcium elevation. 8th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

Poster presentations

Johnson, J.M., Maddula, R., Reichelt, M., Lorenz, S., Schneider, B., Gershenzon, J., Svatos, A. and Oelmüller, R. (2013) Growth and Defense - Ca^{2+} signaling in unstable continuum: *Piriformopsora indica*-derived trisaccharide induces intracellular calcium elevation, promotes growth and confers resistance in Arabidopsis. 12th IMPRS Symposium, Abbe Centre, Beutenberg Campus, Jena, Germany.

Johnson, J.M. and Oelmüller, R. (2011) Role of *Piriformospora indica*-induced intracellular calcium elevation in induced systemic resistance against necrotrophic fungus. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

Johnson, J.M., Seebald, I., Vadassery, J. and Oelmüller, R. (2010) *Piriformospora indica*-induced intracellular calcium elevation confers resistance against necrotrophic fungus in Arabidopsis. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

Johnson, J.M., Seebald, I., Vadassery, J. and Oelmüller, R. (2008) Identification of *Arabidopsis* mutants impaired in calcium response to *Piriformospora indica*. 7th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

9.5 Acknowledgement

I am grateful to all people who have supported and encouraged me during my PhD work. I would first like to express immense gratitude to my supervisor, Prof. Dr. Ralf Oelmüller for his full support, guidance, motivation and inspiring discussions throughout my doctoral study. The financial support from Friedrich-Schiller-University and International Max Planck Research School Jena is greatly acknowledged.

I would like to express my sincere gratitude to my co-supervisor Dr. Ales Svatos for Mass Spectrometry and Dr. Bernd Schneider for NMR, Dr. Dieter Wicher for Ca^{2+} imaging and Prof. Dr. Jonathan Gershenzon for phytohormone, camalexin and glucosinolates studies, at Max Planck Institute for Chemical Ecology; Prof. Frank D. Böhmer, Centre for Molecular Biomedicine for ROS studies; and Prof. Dr. Kirsten Küsel, Institute of Ecology for providing lab facilities to initial work with pathogenic fungi. Along with professors I would like to convey heartfelt appreciation to their group members for cooperation and assistance.

I would also like to extend my gratitude to PhD co-supervisors Prof. Dr. Ian T. Baldwin and Dr. Irena Sherameti. I am particularly thankful to Dr. Irena Sherameti for her constant help. I would like to thank our collaborators of SPP 1212 (DFG) project Prof. Dr. Dierk Scheel, Dr. Justin Lee and Dr. Stefanie Ranf (IPB, Halle). The scientific help from Dr. Klaus Appenroth, Dr. Thomas Pfannschmidt, Dr. Severin Sasso and Dr. Jeannette Pflaz is also greatly acknowledged.

I express my heartfelt thanks to Dr. Ravi, Sybille, Mayuri and Dr. Kai for their help with purification of compounds and Dr. Michael Reichelt for phytohormone, camalexin and glucosinolates studies. I am overwhelmed by motivation, great enthusiasm and hard work of Dr. Michael Reichelt. Thank you very much Dr. Reichelt.

It is my immense pleasure to thank Dr. Jyothilakshmi, Dr. Rinesh and Dr. Pyniarlang for their generous help in the course of the study and wonderful time in Jena. I am particularly grateful for the technical support of Sarah, Claudia and Heide; and the assistance from Alex Kühnl. Thank you Carmen Galambos, Ulrich Weber and Ulrich Möller for all the technical help related to my work in the Institute and stay in Jena. I specially thank Ute, Anne Weiß and Sebastian for their kind help in the completion of thesis.

Furthermore, I would like to thank the support of my former and current lab mates especially,

Dr. Matsuo Matsuhiro, Dr. Thomas Krueger, Dr. Iris, Dr. Yvonne, Dr. Lars, Dr. Sebastian, Yvonne, Eileen, Chao, Anatoli, Madhunita, Anna, Khabat, Matthias, Daniela, Moni, Bjorn Sindy, Doreen, Natalie, Kai and Susane and all the members of the Institute of General Botany and Plant Physiology, Max Planck Institute of Chemical Ecology, Centre for Molecular Biomedicine and the Institute for Ecology.

I extend my gratitude to Dr. Karin Groten, Dr. Carsten Thoms, Mrs. Ulrike Schleier and Katja, coordinators of IMPRS and JSMC for their help in various ways during my PhD studies. Thanks to everyone in the administration at the Friedrich-Schiller-University involved in my contract, and the dissertation procedures, and the stay at Am Herrenberg 11.

I am deeply indebted to Jena Mallus especially Deepesh, Jyothi, Soumya, Abith, Thulasi, Sreejesh, Dhanya, Pavikutty, Rinesh, Xyma, Riyomi, Ramesh, Yamuna, Bhavya, Senthil, Jithin, Amal, Wazeem and Govind; also to Amjath, Rekha and Diyamol. Special thanks to Jena Indians especially Karthik, Prasad and Sagar and their families, Ashok Kumar, Pawan, Amol, Suyog, Glen and Samay. It is a great pleasure to have nice time with the fellow international friends like Theodore, Charlie, Melkamu, Mirid, Tariq, Son and Shipeng, and also with the parish of St. Johannes Baptist Church Jena.

It is my great pleasure to thank Hon'ble Vice-Chancellor, Registrar and Dean (Agri.) of Kerala Agricultural University (KAU), India for the study leave. I would also like to thank all professors and administrative staff at KAU for their kind support. The advice and motivation of Prof. C. Bhaskaran, Prof. Umamaheswaran, Prof. Ganesh, Prof. Gokulapalan, Prof. Anilkumar, Prof. Govindan and also Dr. Rajasekharan sir were of immense help for the completion of my doctoral study. I wish to thank my great friends Dr. Pradeepkumar, Dr. Jacob John, Dr. Jiju P. Alex, Dr. K.D. Prathapan, Dr. K.M. Sreekumar, Dr. Thampan, Dr. Swapna, Mr. Paul and their families for the constant support and encouragement. My special thanks to Sathyan mash, Ruben, Pearson, Rajesh, Sreedevi, Manju and their families for their advice, care and affection.

I have no words to express my gratitude for the affection of my beloved parents, achichan, sister and in-laws, and also the love and care of my niece Bexymol, Bettymol, Riyamol and nephew Ryanmon. Your prayers and wishes helped me a lot during this time.

Above all, I thank The Almighty GOD who made all things possible. YOU are always THE LIGHT shining on my way.